DOCUMENT RESUME

ED 265 080 SE 046 381

AUTHOR	Lim, G., Ed.; And Others
TITLE	Plant Viruses and Mycoplasmas. Proceedings of a Workshop on Plant Viruses and Mycoplasmas Held at the Botany Department, National University of Singapore, Singapore, May 24-27, 1983.
INSTITUTION	Asian Network for Biological Sciences.
REPORT NO	ISBN-9971-62-105-3
PUB DATE	Aug 85
NOTE	129p.
AVAILABLE FROM	Professor A. N. Rao, Secretary, ANBS, Botany Dept., National Univ. of Singapore, Lower Kent Ridge Road, Singapore 0511 ($5.00).
PUB TYPE	Collected Works - Conference Proceedings (021)
EDRS PRICE	MF01 Plus Postage. PC Not Available from EDRS.
DESCRIPTORS	*Botany; Developing Nations; Field Crops; Higher Education; *Microbiology; *Research Methodology; *Scientific Research; Workshops
IDENTIFIERS	Asia; *Mycoplasmas; *Viruses

ABSTRACT

A workshop on plant viruses and mycoplasmas brought together scientists and researchers working on these microorganisms in the countries of eastern Asia, and enabled them to discuss their studies, to exchange ideas, and to become familiar with their counterparts These proceedings of the workshop contain papers which include country reports, current trends of research, and new research methods and techniques. From this 4-day workshop it was found that the research studies on viruses and mycoplasmas are actively pursued in the Asian region. Most of the studies are related to agricultural crops relevant to the countries' economic health, such as rice, pulses, citrus, beans, and coconut among others. Investigations carried out include basic research on structure, identification and classification, as well as methods of controlling diseases and ways of obtaining virus-free planting materials. (JN)

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PLANT VIRUSES AND MYCOPLASMAS

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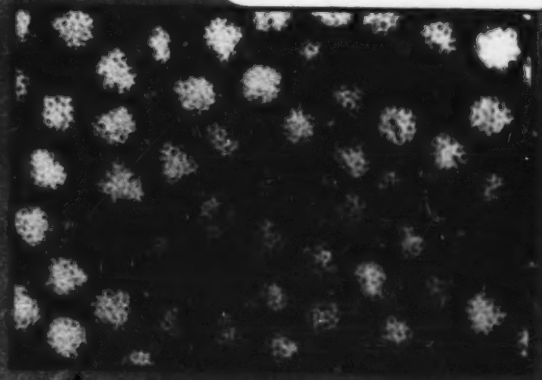

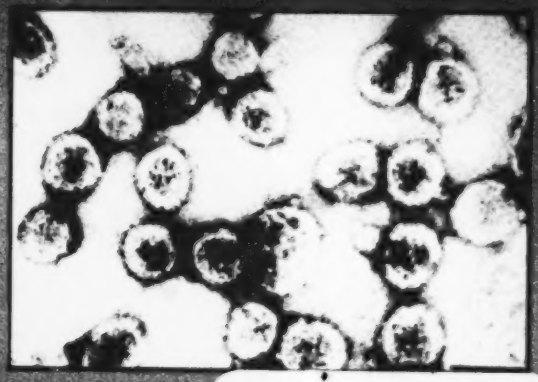

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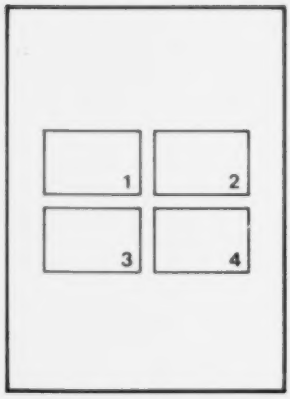

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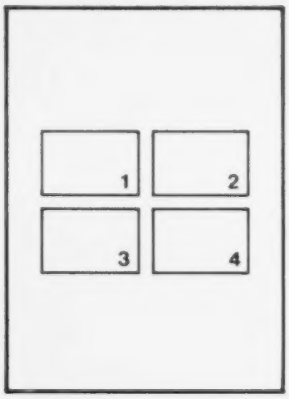

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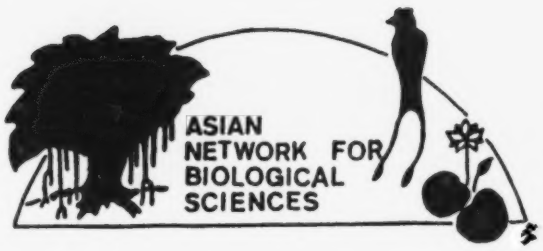
PROCEEDINGS OF A WORKSHOP ON PLANT VIRUSES AND MYCOPLASMAS

Held at the National University of Singapore, Botany Department,

24-27 May 1983

Edited by

G. Lim, A. N. Rao, T. K. Tan and H. H. Yeoh



PROCEEDINGS OF A WORKSHOP ON PLANT VIRUSES AND MYCOPLASMAS

Edited by G. Lim, A. N. Rao, T. K. Tan and H. H. Yeoh

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International Standard Book Number ISBN No. 9971-62-105-3

Printed in Singapore - August 1985

Organised by: Botany Department, National University of Singapore

Published by: Prof A N Rao, Secretary
Asian Network for Biological Sciences (ANBS)

PREFACE

This is the first Workshop on Plant Viruses and Mycoplasmas for South East Asia, organised by the Botany Department, National University of Singapore during 24-27 May, 1983. It was sponsored by UNESCO and COSTED. A special feature of the Workshop was that it brought together scientists and researchers working on these microorganisms in the countries of the Asian region, and enabled them to discuss their studies, exchange ideas and to know their counterparts.

The papers presented were interesting and included country reports, current trends of research, and new methods and techniques practised. From this 4-day Workshop, we found that research studies on plant viruses and mycoplasmas are actively pursued in this region. Much of the studies are in relation to agricultural crops relevant to the countries' economy; crops such as rice, pulses, citrus, beans and coconut among others. Investigations carried out include basic research on structure, identification and classification as well as methods of controlling diseases and ways of obtaining virus-free planting materials.

We hope the papers presented here will be useful to scientists, especially those in Asia.

Editors

G. Lim

A.N. Rao

T.K. Tan

H.H. Yeoh

August, 1985

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RESEARCH AND TRAINING IN PLANT VIROLOGY IN INDIA

M. Raychaudhuri,

Indian National Science Academy,

Bahadur Shah Zafar Marg,

NEW DELHI - 110 002

INTRODUCTION

An early study on plant virus diseases in India was carried out by Pal and Tandon (1937) who established the viral nature of tobacco-leaf-curl and its transmission by Bemisia tabaci. During the last three decades, there has been tremendous advancement in the field of virology. Several research and development work, and training facilities available have made substantial impact on the total agricultural output in India.

This paper briefly describes the various R & D organisations and training in virology, plant infection by viruses and mycoplasma-like organisms and control of virus diseases in India.

R & D INSTITUTES AND UNIVERSITIES ENGAGED IN PLANT VIRUS STUDIES

Several organisations are presently engaged in the study of plant viruses. These include the Indian Agricultural Research Institute (New Delhi), National Botanical Research Institute (Uttar Pradesh), University of Lucknow (Uttar Pradesh), Sugarcane Research Institute (Uttar Pradesh), Central Potato Research Institute (Himachal Pradesh), Tamilnadu Agricultural University (Tamilnadu), Horticulture Research Institute

(Karnataka), National Chemical Laboratory (Maharashtra), Central Rice Research Institute (Orissa) and several others. The areas of virological research cover virus diseases of crop plants, ornamentals, pulses and fruit trees; vector relationship and production of disease-free plants through tissue culture.

VIRUS DISEASES OF CROP AND ORNAMENTAL PLANTS

Various crop and ornamental plants are infected by virus diseases. In most cases, the viruses have been identified, purified and their symptoms and host range described. Their ultrastructural and serological character and vector relationship have also been established. Some of the important virus diseases and plants infected include mosaic of barley, maize, cowpea, soybean, beet, brinjal, potato, banana, papaya, cassava, marigold and petuina; rice grassy-stunt and rice tungro; mosaic streak of wheat; leaf curl of mung, chilli, tomato, papaya and tomato; mottle of Zinnia and Canna; cadang cadang of coconut, ratoon stunting of sugarcane; broken ringspot of tobacco; black ringspot of tomato; leaf roll of potato and bunchy top of banana.

MYCOPLASMA LIKE ORGANISMS (MLO) IN PLANTS

The presence of mycoplasma-like organisms was first reported in 1967 in mulberry dwarf, potato witches broom and aster yellows. All these diseases comprise a group called "Yellows" diseases. These MLO have been found through electron microscopy to have a pleuromorphic cell lacking cell walls and surrounded by triple layered unit membrane, curable by tetracycline and transmitted by leafhopper.

In India, MLO have been reported in several crops such as Apple-Rubbery Wood (Dhingra & Raychaudhuri, 1970); Brinjal little leaf (Varma et al, 1973); Citrus greening (Nariani et al, 1967); Cotton small leaf (Capoor et al, 1972); Potato purple top (Nagaich & Giri, 1973); Rice yellow dwarf (Raychaudhuri et al, 1967); Sandal spike (Varma et al, 1969); Sugarcane grassy shoot (Rishi et al, 1973) and Sesamum Phyllody (Sahambi, 1970).

CONTROL OF PLANT VIRUSES

Several conventional methods such as removal of diseased plants, roguing, rotation of crops, destruction of weeds and vectors are used to control virus diseases.

Virus-free crops of banana, cardamon, strawberry are monitored through, 'seed certification' and 'field certification'. As a result of intensive study of the aphid-vector, seed potatoes are cultivated by coinciding growing period with low aphid population. Other control methods include budwood certification, meristem culture, heat therapy, chemotherapy and use of insecticides against vectors.

Research is also being done to produce virus resistant or tolerant varieties of crops. This may be the best and cheapest method. Virus resistant varieties of wheat and rice have been found.

TRAINING PROGRAMMES

In some of the advanced centres, virology is offered as a major subject leading to M.Sc. and Ph.D. degrees. Short-term training courses are also regularly offered to plant pathologists. Training programmes may be of the following nature:

(1) Technical training on methods of identification of virus diseases, culture collection, inoculation techniques, transmission techniques and knowledge of minor equipment.

(2) 3-6 months training on sophisticated instruments such as the electron microscope, ultra-centrifuge, spectrophotometer and infra-red spectroscope used in advanced virology research.

(3) Short-term training for experts, refresher courses and group discussions on the latest development in virology.

In addition, seminars and conferences are held to enable scientists to present their works. Most of the R & D Institutions in India provide training programmes at various levels.

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PLANT VIRUS AND MYCOPLASMA RESEARCH IN INDONESIA

R. Suseno

Department of Plant Pests and Diseases, Faculty of Agriculture,

Bogor Agricultural University (IPB), Bogor, Indonesia

INTRODUCTION

Studies on plant viruses and mycoplasmas are conducted in various universities and research institutes; e.g. (i) University of Gadjah Mada, Yogyakarta; (ii) University of Padjadjaran, Bandung; (iii) Bogor Agricultural University, Bogor; (iv) Research Institute for Food Crops, Bogor (in cooperation with the Institute for Plant Virus Research, Tsukuba, Ibaraki, Japan, since 1970); (v) Research Institute for Food Crops, Lembang; (vi) Research Institute for Food Crops, Maros; (vii) Research Institute for Sugar Cane, Pasuruan; and (viii) Research Institute for Estate Crops, Jember.

The general areas of study are: (i) identification; (ii) distribution; and (iii) control. Most of the investigations concentrate on virus and mycoplasma diseases of food crops, industrial crops and orchids.

PLANT VIRUSES

The identification of viruses include: (i) symptomatology and host range; (ii) transmission tests; (iii) properties in crude juice; (iv) detection of inclusion bodies by light microscope; (v) interaction with other viruses; (vi) electron microscopy; (vii) purification of the virus; and (viii) serological tests.

Laboratory and field varietal reaction studies are also conducted.

Food Crop Viruses

A. Rice. Three rice virus diseases have been found in Indonesia. These are tungro ("penyakit habang") (Rivera et al, 1968; Tantera, 1973; Saito et al, 1975) grassy stunt ("kerdil rumput")

(Max, 1970; Tantera et al., 1973) and ragged stunt ("kerdil hampa") (Hibino et al, 1977). The tungro virus is found to be transmitted by Nephotettix virescens Dist. and the grassy stunt and ragged stunt virus by Nilaparvata lugens Stal. An outbreak of these diseases occurred in various regions in Indonesia (Tantera, 1973). Drastic replacements of the susceptible varieties by tolerant ones, and controlling the insect vectors are efforts made to reduce the diseases.

B. Leguminous crops. A number of viruses have been found on legume plants in Java and Sumatra.

Peanut: peanut mosaic virus (Thung, 1947; Bergman, 1956), "keriting" virus (rugose leaf curl virus) (Triharso, 1975; Suseno et al, 1982), "kerupuk" virus (Thung & Hadiwidjaja, 1953; Oka, 1981), and peanut mottle virus (Triharso, 1975; Roechan et al, 1978). All the viruses are transmitted by grafting. The insect vector of peanut mosaic virus is Orosius argentatus Evans and that of peanut mottle virus is Aphis craccivora Koch. Peanut mottle virus is also transmitted by seed and is the most important peanut virus. The disease occurs in many localities in Indonesia.

Soybean: soybean stunt virus (Roechan et al, 1975), bean yellow mosaic virus (Roechan 1978), soybean dwarf virus (Iwaki et al, 1975; Suseno et al, 1982), soybean yellow mosaic virus (Roechan et al, 1976), and probably soybean mosaic virus (Roechan et al, 1981). All the viruses are transmitted by Aphis glycines Mats. Soybean stunt and soybean mosaic virus are also transmitted by seed. Soybean dwarf virus is common in various soybean areas in Indonesia.

Cowpea: cowpea aphid-borne mosaic virus (Iwaki et al, 1975; Sulyo, 1981; Suseno et al, 1982), and "kerdil kacang panjang"/"sapu" virus (Semangun, 1958; Suseno et al, 1982). The effect of the latter

virus is more serious than that of the first one. Both viruses can be transmitted by Aphis craccivora.

Bengkuang (Pachyrrhizus erosus Urban): bengkuang mosaic virus (Suseno et al, 1982). The disease is transmitted by grafting, and not by mechanical inoculation. The virus particles are flexuous rods.

C. Potato. Several viruses have been known to occur in Java. These are potato virus X, potato virus Y, potato leaf roll virus, potato virus S, and potato virus M (Anonymous, 1976). All the viruses are transmitted by aphids except potato virus X.

D. Cassava. Cassava mosaic virus is found in Bogor and Pacet (Java) (Bolhuis, 1949; Suseno & Andani, 1975). Formerly the disease was reported to be caused by a drop in temperature (Bolhuis, 1949). Transmission studies revealed that the disease is transmitted by approach grafting, indicating that the disease might be caused by a pathogen, probably a virus (Suseno & Andani, 1975). At a lower temperature the disease showed more severe symptoms.

E. Banana. A banana bunchy-top-like disease has been observed in several locations in West and East Java (Sulyo et al, 1978; Anonymous, 1983). The disease can be transmitted by Pentalonia nigronervosa Coq. At present, further investigations on the disease are being conducted.

F. Citrus. Tristeza virus, psorosis virus and xyloporosis virus have been observed in Indonesia (Thrower, 1959). So far no investigations have been done on these viruses.

Industrial Crop Viruses

A. Tobacco. The most important tobacco virus diseases in Indonesia are tobacco mosaic virus (Breda de Haan, 1898; van der Laan, 1949) and "kerupuk" virus (leaf curl) (Thung, 1932). The "krupuk" virus was reported to be transmitted by Bemisia tabaci (Genn.). The other

viruses such as cucumber mosaic virus (Suseno & Lumanauw, 1972) and pseudomosaic virus (van der Laan, 1949) are confined to certain localities only.

B. Sugar Cane. Three virus diseases have been observed on sugar cane in Java, - sugar cane mosaic virus, chlorotic streak virus and "sembur" virus (Han, 1960). Ratoon stunting was also reported as a virus disease at that time. Investigations were mostly done on sugar cane mosaic virus. This virus is transmitted by mechanical inoculation and its insect vector is Aphis maydis Fitch. Hot water treatment is found to be successful in diminishing the effect of the virus infection on some clones (Handojo, 1976). Gamma rays (Co^{60}) irradiation induced mutants, which have significantly higher resistance against mosaic disease than the original POJ 3016 (Darmodjo, 1976).

C. Orchids

Three viruses, Cymbidium mosaic virus (Suseno, 1978), tobacco mosaic virus - orchid strain (Suseno & Andani, 1975; Suseno, 1978), and Dendrobium mosaic virus (Suseno, 1980) have been found in Jakarta and Bogor (Java). The viruses are easily transmitted by mechanical inoculation.

PLANT MYCOPLASMAS

The identification of mycoplasma-like organisms is based on (i) symptomatology; (ii) transmission tests; (iii) interaction with the vectors, and (iv) electron microscopic studies.

Food Crop Mycoplasmas

A. Rice. Two rice diseases associated with mycoplasma-like organisms are yellow dwarf (Tantera, 1973; Santomi et al, 1978) and orange leaf disease (Tantera, 1973). The former is found to be transmitted by Nephotettix nigropictus Stal. and N. virescens, and the

latter disease by Ricillia dorsalis Motsch. Electron microscopic studies revealed that mycoplasma-like bodies occurred in the sieve tubes of the diseased plants (Saleh et al, 1977; Satomi et al, 1978).

B. Leguminous crops. Several diseases associated with mycoplasma-like organisms on legume plants have been observed in Indonesia.

Peanut: peanut witches' broom. The disease was reported by Thung (1947) as a virus disease. Electron microscopic trials showed that the disease was associated with mycoplasma-like bodies (Saleh et al, 1977; Iwaki et al, 1978; Suseno et al, 1982). The insect vector is Orosius argentatus (Bergman, 1956; Iwaki et al, 1978).

Soybean: soybean witches' broom (Saleh et al, 1977). The causal agent is the same as that of peanut witches' broom.

C. Sweet potato. Sweet potato witches' broom (Saleh et al., 1977). Mycoplasma-like bodies were also observed in the phloem tissues of diseased plants.

D. Citrus. Citrus vein phloem degeneration (CVPD) disease was first reported to be caused by a virus (Tirtawidjaja et al, 1965), but further investigations indicate that the causal agent was a bacterium-like organism (Tirtawidjaja, 1980). The pathogen is transmitted by grafting and its insect vector is Diaphorina citri Kuw. CVPD disease has destroyed most of the Citrus trees in Java and Sumatra. Oxytetracycline treatments (1,000 ppm) improved the growth of the diseased plants.

Plant virus and mycoplasma diseases are becoming more important during the last decade. Hence, it is clear that it will be a task for plant pathologists/virologists to continue with efforts to decrease the

loss of crops caused by plant viruses and mycoplasmas as well as by other kinds of micro-organisms, in order to achieve the best crop production goals expected.

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RICE VIRUSES IN THE TROPICS AND JAPAN

E. Shikata

Department of Botany, Faculty of Agriculture,

Hokkaido University, Sapporo, Japan

INTRODUCTION

Shikata (1979) reported 17 rice viruses, including those known only by their local names. Other reports include rice bunchy stunt in China (Xie & Lin, 1980), witches' broom in Surinam (Ou, 1981) and streak virus in India (Anjaneyulu et al., 1981).

The Fourth Report of The International Committee on Taxonomy of Viruses (Matthews, 1982) described a total of 9 rice viruses as members or possible members of established families, genera or groups.

In this paper, recent findings on the Plant Reovirus Group and a suspected group on rice stripe, rice grassy stunt and maize stripe viruses are presented.

1. Characteristics of Plant Reovirus Group

Rice viruses included in Reoviridae are divided into Plant Reovirus Subgroup 1 (Phytoreovirus) and Plant Reovirus Subgroup 2 (Fijivirus). The main characteristics of these two subgroups are listed in Table 1.

The main differences between the two groups are the genome segments, its molecular weight and the vectors.

Table 2 shows the relationship between Plant Reovirus Group and rice viruses so far known.

The biological nature and structural properties of the virions of rice dwarf virus (RDV), rice gall dwarf virus (RGDV) and rice black-streaked dwarf virus (RBSDV) are better characterised than that of rice ragged stunt virus (RRSV) which lacks the necessary information to determine its taxonomic position.

Table 1. Plant Reovirus Subgroups.

	Phytoreovirus	Fijivirus
Virion	icosahedron double-shelled capsid 65-75 nm in diameter RNA polymerase	icosahedron double-shelled capsid 65-75 nm in diameter RNA polymerase
Genome	dsRNA 12 segments	dsRNA 10 segments
Total molecular weight	16×10^6	$18\text{-}20 \times 10^6$
Polypeptides	7	6
Vectors	leafhopper	planthopper
Localization	phloem or spread	phloem
Symptom	may or may not induce hyperplasia	induce hyperplasia
Cytopathology	globular viroplasm	spread viroplasm

Table 2. Plant Reovirus Group and Rice Viruses.

Plant Reovirus Group	Rice Viruses				
Main characteristics	dwarf	gall dwarf	bunchy stunt	black-streaked dwarf	ragged stunt
icosahedral, double-shelled particles, 65-75 nm	+	+		+	?
dsRNA, 10-12 segments	12	12		10	?
RNA polymerase associated with the capsid	+				
at least 6 different polypeptides	7				
propagative in leafhoppers & planthoppers	L	L	L	P	P
mostly restricted in phloem	more	+		+	+
mostly induce hyperplasia	no	+		+	+
induce viroplasm in the infected cells	+	+		+	+

2. Comparison of rice black-streaked dwarf virus and rice ragged stunt virus

a. Biological properties: Table 3 shows RBSDV and RRSV to have almost similar biological properties.

They are both transmitted by planthoppers. However, the vector of RBSDV cannot transmit RRSV and vice versa, indicating that both viruses are distinct. Host ranges and symptoms are quite similar and sometimes indistinguishable. They cause twisted leaves, swellings on the vein portions of the leaves, sheaths and culms of rice plants. Usually, rice plants infected with RBSDV develop larger swellings than those of RRSV. Nodal branching does not occur in RBSDV infected rice, but appears in RRSV. Symptoms on wheat, barley and maize infected by both viruses are also quite similar. Swellings do not occur on the veins of barley and wheat but they develop twisted leaves, ragged leaves and stunting of the plants. Typical swellings are induced on the leaves of maize infected by RBSDV but not RRSV. Both viruses are restricted to the swellings developed from the phloem or to the phloem cells (Shikata et al., 1979; Shikata, 1981). They induce wide and large viroplasms in the infected cells and are globular in shape, amorphous or scattered in structure. In RBSDV, smaller particles, probably the cores of intact virions, are abundantly scattering within the viroplasm. Intact mature virions with dense cores and less dense outer shells arrange outside the viroplasms or in crevices of the viroplasms. It is not clear if these two particles are present in cells infected with RRSV. So far, it seems that cores or smaller particles are absent in the cells.

Table 3. Biological properties of RBSDV and RRSV.

	RBSDV	RRSV
Laodelphax striatellus	+	-
Nilaparvata lugens	-	+
rice	+	+
wheat	+	+
barley	+	+
maize	+	+
localization	phloem	phloem
tumour	+	+
viroplasm	spread	spread
immature particles in viroplasm	-	+
serological relationship	unknown	unknown

b. Structure of the virions: Intact virions of RBSDV are icosahedron of 70 nm in diameter and consist of double-shelled capsid. The core is about 51-55 nm in diameter surrounded by an outer capsid layer and B-spikes. B-spikes are about 7-8 nm long on the 12 vertices of the icosahedron. A-spikes, about 10 nm in diameter, are attached on each B-spike. The structure of RBSDV is almost identical to that of maize rough dwarf virus, a member of Fijivirus (Milne et al., 1973).

On the other hand, electron micrographs of purified preparations of RRSV revealed that the virions consist of broad based spikes, about 8-10 nm high, 14-17 nm wide at the top and 23-26 nm wide at the base, on the 12 vertices of the icosahedron. The overall diameter of the virions are 57-65 nm. According to Milne (1980), the broad spikes of RRSV are morphologically similar to the B-spikes of Fijivirus. But it seems likely that RRSV does not produce a regular outer layer as do Fijivirus.

c. Genome structure: Boccardo and Milne (1980) first reported that RRSV dsRNA separated into 8 pieces. We obtained 9 bands on 5% and 10%, and 10 bands on 7.5% polyacrylamide gel electrophoresis in Loening's buffer. Recently, it was also reported to separate into 10 pieces (Omura, personal communication). The total molecular weight of RRSV genome, based on RDV dsRNA is 16.2×10^6 daltons.

d. Rice viruses in Plant Reovirus Group: Nine members of Plant Reovirus Group are divided into 2 subgroups based on the vectors and the genome structures (Table 4). Boccardo and Milne (1980) separated RRSV genome into 8 pieces and calculated total molecular weight of 11.36×10^6. It was suggested that RRSV be placed away from subgroups I & II (Matthews, 1982). The present studies showed RRSV has the same number of dsRNA segments as Fijivirus. However, total molecular weights of RRSV genome

Table 4. Plant Reovirus Group.

Virus	No. dsRNA segments	Main host	Main vector
Subgroup I, Phytoreoviruses			
Clover wound tumour	12	Trifolium incarnatum	Agalliopsis novella
Rice dwarf	12	Oryza sativa	Nephotettix cincticeps
Rice gall dwarf	12	O. sativa	N. nigropictus
Subgroup II, Fijiviruses			
Sugarcane Fiji disease	10	Saccharum officinarum	Perkinsiella saccharicid
Maize rough dwarf Cereal tillering disease	10	Zea mays	Laodelphax striatellus
Rice black-streaked dwarf	10	O. sativa	L. striatellus
Pangola stunt	10	Digitaria decumbens	Sogatella frucifera
Oat sterile dwarf Arrhenatherum blue dwarf Lolium enation	10	Avena sativa	Javesella pellucida
Ungrouped			
Rice ragged stunt	10	O. sativa	Nilaparvata lugens

are somewhat larger than those reported for Fijivirus. In addition, whether RRSV has double-shelled capsid or not needs to be confirmed. Therefore, RRSV still remains "ungrouped" in Plant Reovirus Group (Table 4).

Rice bunchy stunt was shown to be transmitted by Nephotettix cincticeps and N. virescens. The virus did not react with RDV antiserum. The virus-like particles in ultra-thin sections of diseased plants are 35-60 nm in diameter and appears smaller than Phytoreovirus (Xie & Lin, 1980). According to Xie & Lin (1980), rice bunchy stunt virus is a new virus. It needs further confirmation on particle morphology and genome structure before it can be included in the Phytoreovirus.

2. Relationship among rice stripe, rice grassy stunt and maize stripe viruses

a. Biological properties: Rice stripe virus (RSV) and rice grassy stunt virus (RGSV) are propagative viruses specifically transmitted by planthoppers, Laodephax striatellus and Nilaparvata lugens respectively. RSV has a wide host range, whereas RGSV infects only rice (Senboku & Shikata, 1980), with more or less chlorotic streaks on the leaves appearing as symptoms. Maize stripe virus (MStV), propagatively transmitted by Peregrinus maidis, infects maize and barley, but not wheat, showing chlorotic streaks on the leaves. The three viruses were reported to be small spherical particles (Kitani & Kiso, 1965; Kulkarni, 1973; Shikata & Senboku, 1980), but later studies (Koganezawa et al., 1975; Toriyama, 1982) suggest fine filaments as the infective agents of these viruses. At present, the fundamental structure of the RSV agent is not clearly determined.

Gingery et al. (1981) detected an abundance of fine threads, about 3 nm in width, in the purified preparations of maize plants infected with MStV, but these were not associated with infectivity.

Hibino et al. (1982) recently observed small amounts of spherical particles, 18-20 nm in diameter and larger amounts of fine loop filaments, about 2,000 x 5-20 nm, in non-infective purified preparations of RGSV. They showed that the structure of RGSV filaments is similar to those of RSV.

Serological relationship was shown between filaments of purified RSV preparations and the anti-MStV filaments by agar gel diffusion (Yamashita et al., 1981). RSV filament preparations also gave positive serological reactions with anti-RGSV fine filaments (Hibino et al., 1983).

c. Microprecipitin test and immune electron microscopy of rice stripe virus filaments and the antiserum against maize stripe virus: Microprecipitin tests between RSV slender loop filaments and MStV-antiserum indicated that the titers obtained by RSV and MStV-antiserum were almost the same as those obtained by RSV and RSV-antiserum (Table 5). Immune electron microscopy showed apparent serological reaction in the mixtures of the slender loop filaments of RSV and MStV-antiserum, as well as RSV-antiserum.

d. Suspected maize stripe virus group: Although the fundamental structure of the 3 viruses responsible for their infectivity are not clearly determined, some biological properties and serological relationship of the filaments from the 3 viruses suggest that a group including RSV, RGSV and MStV may be formulated (Table 6).

Table 5. Microprecipitin tests of rice stripe virus.

Reciprocal of antigen dilution[a]	Reciprocals of antisera dilution													
	Rice stripe virus antiserum							Maize stripe virus antiserum						
	4	8	16	32	64	128	PB[b]	4	8	16	32	64	128	PB
2	++	+	+	-	-	-	-	+++	++	++	+	-	-	-
4	++	+	+	+	-	-	-	++	++	++	+	±	-	-
8	++	+	+	+	-	-	-	++	++	++	+	±	-	-
16	+	+	+	+	±	-	-	+	+	+	+	±	-	-
32	+	+	+	+	±	-	-	+	+	+	+	±	-	-
PB	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a) Purified rice stripe virus: undiluted sample had A_{260} of 0.6.

b) 0.01 M phosphate buffer.

Table 6. Maize stripe virus group.

Viruses	Main hosts	Principal vectors	Ovary trans.	Virus particle	Serol. relation
Maize stripe	maize barley (wheat)	Peregrinus maidis	+	3 nm thread	o
Rice stripe	rice maize barley wheat	Laodelphax striatellus	+	400 x 8 nm branched filament 3 nm circular thread	o o
Grassy stunt	rice	Nilaparvata lugens	-	1,000-3,000 x 6-8 nm circular thread	o
Wilted stunt	rice	N. lugens			
Hoja blanca	rice wheat rye	Sogatodes orizicola	+	fine thread	

e. Rice hoja blanca and wilted stunt viruses: Rice hoja blanca virus is also propagatively transmitted by planthoppers (Galvez, 1967). The causal agent in purified preparations has not been found. In ultra-thin sections of infected cells of rice plants, abundant thread-like structures, suspected to be the agent of this virus, were detected (Shikata & Galvez, 1969). These threads were found in the nuclei and cytoplasm of infected phloem, mesophyll and epidermal cells.

Rice wilted stunt disease was reported in Taiwan as a new virus disease transmitted by Nilaparvata lugens, the same vector for RGSV (Chen et al., 1978). Symptoms on rice are almost identical but more severe than those of RGSV. It could be a severe strain of RGSV.

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VIRUS AND MYCOPLASMA DISEASES IN THAILAND

D. Choopanya

Virology Section, Division of Plant Pathology and Microbiology

Department of Agriculture, Bangkokhen *10900, Bangkok, Thailand

INTRODUCTION

Increase in agricultural acreage during the past decade of the country has lead to more disease problems. Thailand is one of the countries where one third of her income is from exporting agricultural products. The main crops of the country are rice, cotton, corn, cassava, sugar cane, pineapple, soybean, mungbean, groundnuts, orchids, nulburry and vegetables etc.

In the past only a few diseases and insects were concerned, but today the introduction of different varieties of crops brings new pathogens and insects into the country. The growers use chemicals such as fungicides and insecticides which in turn lead to micro organism and insects becoming tolerant to chemicals. So more diseases become widespread in the growing areas especially diseases caused by viruses and mycoplasmas.

Virus and mycoplasma diseases of economic crops in Thailand

1. Rice ragged stunt virus

This virus was first observed in 1977. Symptoms are stunting, twisted leaves, ragged leaves, incomplete emergence of panicles and vein-swelling on the outer surface of leaves and leaf sheaths.

The virus is not transmitted through seed or soil. Transmission tests by insect vectors found in paddy fields, Nilaparvate lugens Stal, Nephotettix virescens Distant, Nephotettix nigropictus Stal. Recillia dorsalis Motschulsky, and Sogatella furcifera Norvath, were conducted by allowing them to feed directly on diseased plants and then were inoculated to healthy rice seedling grown in insect proof greenhouse.

Only the brown planthopper, Nilaparvata lugens Stal could transmit the disease in a persistent manner without transvorial passage. The shortest acquisition feeding period was 2 hours, the shortest inoculation period was 1 hour and the incubation period in insect vectors was 8 days an average. Transmissibility of the disease by nymph and adult Nilaparvata lugens Stal was examined. The fourth instar nymph and the fifth instar nymph showed the highest percentage of transmitters. There was no difference in incubation period between each instar of nymph and adult. Spherical virus particles of about 60 nm in diameter were found in dip preparation from vein-swelling and leaves infected with the disease. Electron micrographs revealed abundant polyhedral particles in phloem cells about the same size.

This disease was severe in several provinces in Thailand. It is similar to "Ragged stunt" in the Philippines and "kerdil Hampa" in Indonesia.

2. Rice transitory yellowing virus

It was first observed in the northern part of Thailand in 1979. The disease is characterized by slight to severe stunting of the infected plants, orange-red discolouration of leaf blade usually starting from the leaf tip and gradually advancing downwards. The yellow discolouration is more intense in the lower leaves than in the upper ones.

Bullet-shaped particles, belonging to the shabdo virus group, 86-94 nm wide, 120-130 nm long, were observed in negatively stained preparations of diseased plants. In ultra-thin sections of infected rice leaves, bullet-shaped particles, 83-89 nm wide, 188-200 nm long, were observed in phloem cells. The particles were closely associated with the nuclear membrane of the cell. Many particles dispersed randomly in the

cytoplasm of infected cells were also observed. The cells containing the virus particles often showed necrosis. In the nuclei of the infected cells, chromatin substances were reduced and the nucleoplasm had a fine, uniform appearance.

The disease was transmitted in a persistent manner by leafhoppers. The incubation period in insects and in plants is 13-15 days, about 14 days respectively. The viruliferous leafhoppers could often retain their infectivity for their entire life span. The efficiency of transmission differs by species e.g. Nephotettix nigropictus (75%), N. cincticeps (59%), N. virescens (5%).

3. Rice gall dwarf

Rice gall dwarf is a new rice virus disease found in Uthai Thani Province, in the northern part of the central plain of Thailand in August, 1979.

Major symptoms of this disease are stunting, dark green in leaf colour and small galls on leaf blades and sheaths. Symptom development on rice seedlings inoculated at the young growth stage was as follows: about two weeks after inoculation, stunting and dark green leaf colour was observed, and 3 to 4 days after showing stunting, galls appeared on the outer surface of leaf blades and they extended to the outer surface of leaf sheaths. Galls were pale green to translucent white, and extended along leaf veins. Each gall was short in length and did not become streaks of vein swelling. Heading was delayed and the number of tillers was decreased.

Transmission was by Nephotettix nigropictus and Recilia dorsalis. The incubation period in the insects was 8 days on average and the virus was transmitted in a persistent manner. Spherical particles about 65 nm

in diameter were observed by the electron microscope in dip preparations from infected rice plants.

The occurrence of rice gall dwarf disease has been shown in 7 provinces in Thailand, they are Uthai Thani, Chainat, Sukhothai, Chachoengsao, Bangkok (Bangkhen), Pathom Thani, and Nakorn Pathom.

4. Cotton leaf roll

Cotton (Gossypium hirsutum) in the major growing areas in Thailand was found to have symptoms similar to those described for cotton leaf roll disease. These symptoms include stunting, shortened internodes, curling and brittle and shiny leaves with always vein clearing of young leaves. The causal agent was transmitted from an infected cotton plant to a healthy one by grafting, but it was not transmisible through seeds or by mechanical inoculation. The causal agent was readily transmitted by the cotton aphid (Aphis gossypii) in a persistent manner with a latent period of 2.5 to 6 days. The minimum feeding period for A. gossypii to acquire the causal agent was between 3-6 hours. The inoculation period of the causal agent within the cotton plant ranged from 12-20 days. The increase in the number of aphids feeding on each seedling increased transmission efficiencies; Sida acuta, a common weed in cotton fields is susceptible to the causal agent.

5. Peanut mottle virus

Peanut mottle virus (PMV) is a very common and widespread disease of groundnut in Thailand. The virus is transmitted mechanically, by grafting and by the aphid, Aphis craccivora Koch. Chenopodium amaranticolor and C. quinoa are local lesion hosts. Systemic mottle symptoms were induced in azuki bean (Phaseolus angularis), beans (P. vulagaris) 'Nanus', 'Berta' and 'Tsurunashi kintoki', cowpea (Vigna

unguiculata), peanut (Arachis hypogaea) and soybean (glycine max).

The flexuous rod particles were observed both in purified preparations and in dipping infected leaves. The virus particles are approximately 760 nm. In sections of diseased leaves, pinwheel inclusions and platelike structure were observed. No such structures were found in healthy tissues.

The thermal inactivation point of the virus is between 55 and 60°, dilution end point between 10^{-3} and 10^{-4}, and infectivity is retained in crude sap after 18 hr but not 24 hr at 27°C.

6. Soybean yellow vein virus

A yellow disease of soybean was isolated from a soybean plot in central Thailand. The disease symptoms could be induced by grafting or mechanical transmission to healthy soybean plants. Among the many plant species tested by mechanical inoculation, only soybean and Chenopodium amaranticolor were susceptible to the disease. Inoculated soybean plants showed patches of yellow veins on newly emerging trifoliate leaves at 30 days after inoculation. The number of these yellow patches later increased making the whole leaf yellow and necrotic. Inoculated leaves of C. amaranticolor showed local chlorotic lesions 6 to 7 days after inoculation. Electron microscopy revealed rod-shaped particles (20 x 500 nm) in leaf-dip preparations of infected leaves of soybean or C. amaranticolor. In ultra-thin sections, similar particles were detected in infected but not healthy soybean cells. These particles appeared either randomly or as stacks of parallel particles in the cytoplasm. Both the particles in leaf-dip preparations and ultra-thin sections appeared to be a tobamovirus.

7. Mungbean yellow mosaic

Yellow mosaic disease of mungbean in Thailand is caused by a whitefly-transmitted agent, presumably a virus. Ultrastructural changes similar to those previously reported for whitefly-transmitted viruses are associated with the disease. The virus-like particles (VLPs) associated with the disease were isometric, about 15-20 nm in diameter, and often formed loose aggregates that sometime almost filled the total nuclear volume of infected phloem cells. Mungbean infected by whitefly transmission or by grafting had hypertrophied nucleoli, aggregates of VLPs, and fibillar bodies in the nuclei of phloem cells as early as 2 days before symptom appearance. In the vacuoles of lumen of the partially or fully differentiated infected sieve elements, VLPs occasionally formed aggregates having a double cylindrical arrangement of particles. No VLPs were defected in tissues other than the phloem of infected plants or in any tissues of comparable healthy plants.

8. Fern-leaf of tomato

A virus disease causing fern-leaf symptoms on tomato was observed in almost all tomato planting areas in Thailand. The virus particle was icosahedral, about 30 nm in diameter. It has a very wide host range, infecting 36 species of plants in 7 families. Important host plants are cucumber (*Cucumis sativus* L.), tobacco (*Nicotiana* spp.), pepper (*Capsicum* spp.), cowpea (*Vigna sinensis* Savi), *Chenopodium amaranticolor* Coste & Reyn Willd. Its physical properties are: thermal inactiviation point between 60-65°C, dilution end point between 10^{-4} - 10^{-5} and longevity *in vitro* between 12-15 hours at 23-26°C. Thus this virus is related to cucumber mosaic virus.

9. Papaya ring spot

A virus causing severe mosaic, leaf malformation, and ringspot symptoms in papaya was isolated from Northeastern Thailand. The virus which was transmisable by Aphis craecivora Koch. in a non-persistent manner has flexuous rod particles (10 x 750 nm) and induces cylindrical and pinwheel inclusions in the cytoplasm of infected papaya cells. Properties of the virus in plant sap include a thermal inactivation point of 55°C, a dilution end point of 10^{-3}, and a longevity in vitro of 7 hrs at room temperature. The virus induced local necrotic lesions on inoculated leaves of Chenopodium amaranticolor and systemically infected papaya (Carica papaya), pumpkin (Cucumis moschata) (Duch.) Poir, but not Cassia occidentalis and Gomphrena globosa which are hosts of papaya mosaic virus. Based on these properties the virus isolated from infected papaya plants in Thailand was identified as papaya ringspot virus.

Mycoplasma-like organisms

Sesame phyllody: A phyllody disease of sesame (Sesamum indicum) was reported from Northeast Thailand. The characteristic symptoms are stunted growth, extreme proliferation of the growing tip, and numerous small leaves, giving the infected plant a witches-broom effect. Part or all of the floral parts are transformed into green leaflike structures, and little or no seed is produced. Electron microscope studies showed the presence of mycoplasma-like bodies in diseased but not in healthy tissue.

Phyllody of soybean, mungbean, peanut and wingbean: In 1980-1981 mycoplasma-like organisms spherical to pleomorphic bodies of 80-800 nm, were found in the phloem elements of the four kinds of plants in Leguminosae which showed phyllody symptoms-soybean (Glycine max (L.)

Merr.), mungbean (*Phaseolus aureus* Roxb.) peanut (*Arachis hypogaea* L.), and wingbean (*Psophocorpus tetragonolobus*). No such bodies were observed from healthy tissues.

COMPARATIVE STUDIES ON HOP STUNT VIROID, CUCUMBER PALE FRUIT VIROID AND POTATO SPINDLE TUBER VIROID

T. Sano, *M. Sasaki, I. Uyeda, **M. Kojima and E. Shikata

Department of Botany, Faculty of Agriculture, Hokkaido University, Sapporo, Japan; *Kirin Brewery Co. Ltd, Tokyo, Japan; **Laboratory of Plant Pathology, Faculty of Agriculture, Niigata University, Niigata, Japan

INTRODUCTION

Hop plants are cultivated in many regions of the world in relation to beer production. The plants cultivated in Japan originated from European cultivars. However, hop stunt disease, so far as we are aware, has been reported only in Japan.

Most Japanese commercial hops (Humulus luplus L.) are infected with a complex of several viruses, showing mosaic or latent symptoms.

Typical symptoms of hop stunt disease are shortening of the internodes of the main and lateral vines and curling of the upper leaves. The plants become severely stunted and thus the name of the disease.

The main vines of the plants which are free from the disease usually grow to about 8 m high, whereas infected ones with same number of nodes grow to only about 5 m high.

In 1970, Yamamoto et al reported that the disease agent was mechanically transmissible to hop and wild hop (Humulus japonicus Sieb. et Zucc.), and spread along the ridges of hop gardens. Following experiments involving grafting, sap transmission, symptomatology and epidemiology, they concluded that the disease was caused by a virus, although no causal virus particles were detected in the diseased plants.

The cone yields of the diseased hops were reduced approximately to half of those of stunt-free hops. In addition, the -acid content in the bitter substances in cones from diseased hop plants was reduced about one half to one third of that from stunt-free hops.-

Sasaki and Shikata (1977 a & b) found that hop stunt disease was able to infect cucumber plants by mechanical inoculation, and showed severe symptoms. Using cucumber plants, they indicated that its infectivity was closely associated with a low molecular weight RNA, about 7S RNA fraction, and the disease agent was DNase insensitive but RNase digestive. Finally they concluded that the causal agent of the disease was a viroid.

The host plants of hop stunt viroid (HSV) are restricted to hops, cucurbitaceous plants and tomatos. Symptoms expressed on the infected plants seemed to be identical with those infected with cucumber pale fruit viroid (CPFV) in Netherlands, (van Dorst & Peters, 1974).

Therefore, we carried out some comparative studies of both viroids.

RESULTS AND DISCUSSION

The following comparisons of HSV, CPFV and potato spindle tuber viroid (PSTV) were made:

I. Biological properties in host range and symptomatology

II. Cytopathic changes in ultrathin section

III. Co-electrophoresis of the partially purified preparations

1) 15% PAGE, non-denaturing condition

2) 7.5% PAGE, denaturing condition

3) 5% PAGE, denaturing condition

IV. Estimation of number of nucletoides of purified viroid preparations.

V. Electron microscopy of purified viroid preparations

1) non-denaturing condition

2) denaturing condition

I. Biological properties in host range and symptomatology

HSV, CPFV and PSTV were inoculated on 11 test plants belonging to Cucurbitaceae, Solanaceae and Compositae. The results are shown in Table 1.

Both HSV and CPFV developed symptoms on cucumber (Cucumis sativus), melon (C. melo), Benincasa hispida, Lagenaria siceraria var. clavata and L. siceraria var. microcarpa. Cucumber plants infected with HSV and CPFV showed stunting, vein clearing and leaf curling 14-17 days after inoculation. The other 4 plants in Cucurbitaceae also showed stunting, leaf curling and top necrosis 16-22 days after inoculation, and the severely infected plants sometimes died. Tomato plants caused symptomless infection by HSV and CPFV. Watermelon (Citrullus vulgaris), squash (Cucurbita maxima) and Gynura aurantiaca were not infected with HSV and CPFV. It was shown that host ranges of HSV and CPFV were almost identical. Moreover, the symptoms on each test plant were similar and thus it seemed difficult to differentiate between HSV and CPFV by their biological properties.

On the other hand, tomato plants infected with PSTV developed severe stunting and epinasty about 14 days after inoculation, but cucumber, melon B. hispida, L. siceraria var. clavata, L. siceraria var. microcarpa, watermelon and squash were not infected with PSTV. Thus PSTV was apparently biologically different from HSV and CPFV.

Table 1. Sap inoculation experiments

Plants	Symptoms[a]		
	HSV	CPFV	PSTV
Benincasa hispida	LC, M, St, TN, D	LC, M, St, TN, D	-(0/2)[c]
Citrullus vulgaris (Shimatama suika)[b]	-(0/6)	-(0/6)	-(0/2)
Cucumis melo (Yubari melon)	LY, N, St, D	LY, N, St, D	-(0/2)
C. sativus (Suyo)	LC, VC, St	LC, VC, St	-(0/2)
(Sporu)	LC, VC, St	LC, VC, St	-(0/2)
Cucurbita maxima (Ebis)	-(0/6)	-(0/6)	-(0/2)
Lagenaria siceraria var. clavata (Daimaru)	LC, St, M, TN, D	LC, St, M, TN, D	-(0/2)
L. siceraria var. microcarpa (Sennari hyoutan)	LC, St, M, TN, D	LC, St, M, TN, D	-(0/2)
Lycopersicon esculentum (Rutgers)	1 (3/3)	1 (3/3)	St, Ep
(Rentita)	1 (3/3)	1 (3/3)	St, Ep
Gynura aurantiaca	- (0/3)	- (0/3)	1 (2/2)

a) LC: Leaf curling. M: mosaic, St: stunting. TN: top necrosis. D: plant death. LY: leaf yellowing. N: necrosis. VC: veinclearing. I. symptomless. Ep: epinasty.

b) cultivar name.

c) results of back-inoculation; numbers of plant infected/inoculated.

II. Cytopathic changes in ultrathin sections

1) Cucumber plants infected with HSV and CPFV

Symptoms caused by HSV were quite similar to those caused by CPFV in cucumber plants. Each cucumber plant infected with HSV and CPFV showed very severe symptoms such as yellowing and shortening of internodes. Table 2 summerizes the cytopathic changes caused by both viroids in the infected cucumber plants, and indicates that their internal symptoms were almost identical. One of the typical alterations was aberration of cell walls in all tissues, such as epidermis, palisade parenchyma, spongy parenchyma and vascular bundles. The cell walls of HSV- and CPFV-infected cucumber leaves were extremely distorted. These areas were characterized by cell walls with corrugated profile and irregular thickness. These aberrations appear to lead to a structural deformation of the tissues themselves, resulting in external symptoms. Another alteration in the infected leaf cells was disintegration of the chloroplasts. Furthermore, these were usually accompanied by breakdown of tonoplast and production of granules within the cytoplasm. These structural changes were almost the same as those of tomato plants infected with PSTV and citrus exocortis viroid (CEV).

2) Tomato plants infected with HSV and CPFV

Tomato plants showed no symptoms when infected with HSV and CPFV, but proved capable of carrying these agents. Therefore, it was rather difficult to find out distinctive alterations of leaf cells in the infected tomato plants. However, disintegration of chloroplasts sometimes, but not often, occurred in these systems.

III. Co-electrophoresis of the partially purified preparations

In preliminary experiments using nucleic acid extracts from HSV-,

Table 2. Cytopathic changes in cucumber and tomato plants infected by viroids.

Host	Cucumber (Suyo)			Tomato (Rentita)				
Viroid	HSV	CPFV	control*	HSV	CPFV	PSTV	CEV	control*
External symptom	++	++	-	-	-	++	+++	-
Papamural body	+	+	+	+	+	+	+	+
Aberration of cell wall	++	++	-	-	-	++	++	-
Chloroplast disintegration	++	++	-	+	+	+++	+++	-
Disappearance of tonoplast	+	+	-	+	+	+	+	-

* healthy plants

CPFV-, and PSTV-infected tomatos, it was shown that migration rates of HSV and CPFV were almost identical on 5% polyacrylamide gel. In addition, they displayed identical migration rates with cellular RNA (7S RNA) and thus no viroid specific band was observed. On the other hand, the extract from PSTV infected tomatos displayed a specific band associated with infectivity.

IV. Estimation of number of nucleotides of the purified viroid preparations

The result of the bioassay from the two components of each viroid preparation indicated that both components were highly associated with infectivity. It was assumed that these two components were circular and linear molecules of the respective viroids (Sanger et al, 1979; Ohno et al, 1982). The number of nucleotides of linear molecules of HSV and CPFV were estimated using those of PSTV, 5S rRNA and tRNA as markers and calculated to about 315 nucleotides and about 320 nucleotides for HSV and CPFV, respectively.

V. Electron microscopy of the purified viroid preparations

1) Non-denaturing condition: The preparations for electron microscope examinations were made according to the method described by Sogo et al. (1973).

Under non-denaturing condition, short-rod like molecules about 50 cm long, which seemed to be native viroid molecules were observed. In this condition, viroid molecules aggregated to each other.

2) Denaturing condition: The purified preparations of HSV and CPFV were spread on water hypophase according to the method described by Randles & Hatta (1979) with some modifications. The viroids (2 ug/10 ul) were dissolved in 90 ul of a formamide solution (99% formamide-10 mM

Tris, pH 8.5-1 mM EDTA). The mixture was then heated at 60°C for 15 min. Immediately after heating, cytochrome C (4 ug/4 ul) was added and the mixture was spread on hypophase (1.5 mM Tris, pH 8.5-0.15 mM EDTA) preheated at 60°C, picked up on the grids and stained.

Under denaturing condition, most of the molecules observed were closed circular forms. The linear forms were scarcely observed in both HSV and CPFV preparations. The result complemented that of Sanger et al (1976). They examined PSTV by electron microscopy under strongly denaturing condition, and indicated that most of the viroid molecules were closed circular form and linear ones constituted less than 1%.

The length of viroid molecules was calculated from circular molecules under denaturing condition. The mean length of HSV and CPFV were 80-100 nm and 90-100 nm, respectively. It was difficult to distinguish both viroids by electron microscope examinations.

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A RECENT METHOD FOR IDENTIFYING AND CLASSIFYING VIRUSES USING COMPLEMENTARY DNA

N. Abdul Samad

Department of Biochemistry and Microbiology

Faculty of Science and Environmental Studies,

Universiti Pertanian Malaysia, Serdang, Selangor, Malaysia.

INTRODUCTION

Molecular hybridization is a technique which involves the determination of the rate and extent of the formation of base-paired nucleic acid duplexes from their component strands. The several classes of hybridization reactions include reassociation of denatured double-stranded DNA; hybridization of a nucleic acid species immobilized on a filter to complementary strands in solution (either RNA or DNA); and hybridization of cDNA transcribed in vitro from an RNA template, to the template RNA or other RNA species.

The third class of hybridization reaction has been used with plant viruses in recent years. The random primed synthesis of cDNA permitted a comparison of the sequences of the RNA component of cucumber mosaic virus (CMV) (Gould & Symons, 1977) and alfalfa mosaic virus (AMV) (Gould & Symons, 1978) and allowed comparisons between CMV isolates (Gonda & Symons, 1978), members of the tymovirus group (Kummert & Kettman, 1978), tobamovirus group (Palukaitis & Symons, 1980) as well as between pairs of viruses with otherwise similar properties (Gould & Hatta, 1981; Mohamed & Mossop, 1981).

MATERIALS AND METHODS

Synthesis of Complementary DNA

The method of Taylor et al. (1976) as modified by Gould & Symons (1977) was used to synthesize cDNAs to 3 isolates of BYMV and WMV. BYMV-RNAs were primed with oligodeoxyribonucleotides generated by DNase treatment of salmon sperm DNA (Taylor et al., 1976). The

oligonucleotides annealed with randomly occurring complementary sequences on the RNA template and acted as primers for cDNA synthesis with avian myeloblastosis virus reverse transcriptase.

cDNA to WMV-1 was prepared by the same procedure except that herring sperm DNA was used as the primer and [^{3}H] dCTP was used at a specific activity of 18.5 Ci/mmole.

RNA-cDNA Hybridization

(a) Hybridization Procedure

The hybridization buffer was 0.01M Tris-HCl pH 7.0, 0.18M NaCl, 1 mM EDTA and 0.05% SDS (Gould & Symons, 1977). The reaction mixtures (40 µl) contained purified viral RNA and [^{3}H]-cDNA (ca. 2,000 cpm). Mixtures were heated at 100°C for 2-3 mins. and incubated at 65°C. Hybridization was terminated by cooling the tubes on ice and processed immediately or stored at -20°C.

The $R_o t$, cocentration of ribonucleotides at zero time multiplied by time of hybridization, value was calculated as:

$$R_o t = \frac{\text{RNA concentration x hybridization time}}{\text{Molecular weight of ribonucleotide}} \quad \text{mol sec litre}^{-1}$$

where RNA concentration is expressed as mg/ml, the hybridization time in seconds, and the average molecular weight for the ribonucleotides assumed to be 320.

(b) S_1 Nuclease Assay for Extent of Hybrid Formation

30 µl samples of the hybridization reaction mixtures were added to S_1 assay buffer pH 4.6 containing 0.03M sodium acetate, 0.05M NaCl, 1 mM $ZnSO_4$, 5% glycerol, and 40 µg/ml of denatured calf thymus DNA. Two 150 µl samples were taken and to one was added 2 units of S_1 nuclease while

the other was left as a control. Both were incubated at 45°C for 30 mins and terminated by addition of 1.0 ml of 10% TCA and 75 µg of bovine serum albumin. After 30 mins. at 4°C the TCA precipitable material was collected by filtration through GF/A glass-fibre filters (Whatman), washed with TCA and ethanol, dried and radioactivity determined.

(c) Calculation of Estimated Percentage of Homology

The extent of hybridization, measured by the S_1 nuclease assay, was estimated as the % S_1 nuclease resistance which was:

$$\frac{\text{c.p.m. in aliquot treated with } S_1}{\text{c.p.m. in aliquot treated without } S_1}$$

Values were corrected for self-annealing of the cDNA in the absence of RNA as follows:

$$\text{Corrected percentage } S_1 \text{ nuclease resistance} = 100 \times \frac{S_1 \text{ nuclease resistance of hybrid (\%)} - S_1 \text{ nuclease resistance of cDNA(\%)}}{100 - S_1 \text{ nuclease resistance of cDNA (\%)}}$$

The estimated percentage homology was then calculated by dividing the corrected percentage S_1 nuclease resistance of the heterologous hyrbid by the corrected percentage S_1 nuclease resistance of the homologous hybrid and multiplying by 100 (Gonda & Symons, 1978; Palukaitis & Symons, 1980).

$$\text{i.e. } \frac{\text{\% hybridization (heterologous)}}{\text{\% hybridization (homologous)}} \times 100$$

RESULTS

Hybridization between BYMV isolates

The relationships between three biologically distinct isolates of BYMV, which differed in their original hosts and their site of occurrence

(Randles et al., 1980; Abu-Samah & Randles, 1981) were studied using cDNA. Reciprocal hybridizations between BYMV-G, BYMV-Q and BYMV-S (Table 1) showed that each was significantly different from the other. BYMV-G and BYMV-Q were more closely related and BYMV-S more distantly related, but with higher homology to BYMV-Q. While it is not yet possible to relate these differences to the biological properties which are known, it is clear that molecular hybridization analysis showed a larger difference between BYMV-Q and BYMV-S.

Studies of Homology between WMV-1 and WMV-2

As all attempts to synthesize the cDNA to WMV-2 were unsuccessful, the relationship between WMV-1 and WMV-2 was determined by using the cDNA synthesized to WMV-1 only. Heterologous hybridizations between WMV-2 and WMV-1 cDNA (Table 2) showed that there was no detectable homology between the two. Therefore, these data confirmed that WMV-1 and WMV-2 were not related and were distinct under the conditions used.

DISCUSSION

Although the mechanism involved in molecular hybridization analysis (MHA) is not fully understood, it has been shown to be useful for looking at relationships within subgroups particularly for the potyviruses where other criteria are not useful. It was found that significant homology was demonstrable only between potyviruses which are closely related. For example, the -G, -Q and -S isolates of BYMV which are closely related serologically and biologically (Randles, et al., 1980; Abu-Samah & Randles, 1981) were distinguished quantitatively by their percentage homology. Conversely, WMV-1 and WMV-2, which are

TABLE 1 : Estimates of homology between BYMV-G, BYMV-Q and BYMV-S

RNA	Percentage homology with cDNA to G	Q	S
G	83 ± 5.2[a](100)[b]	70 ± 5.1 (84)	18 ± 0.9 (22)
Q	65 ± 3.9 (84)	77 ± 4.7 (100)	31 ± 0.9 (40)
S	14 ± 3.4 (16)	34 ± 2.3 (41)	83 ± 4.5 (100)

[a] Percentage homology corrected for self-hybridization of cDNA. Standard error of the mean of three replicates for each hybridization reaction is shown.

[b] Values in parenthesis are calculated assuming 100% for the homologous reactions.

TABLE 2 : Hybridization specificity of WMV-1 cDNA

Nucleic acid	RNA Concentration (ug/ml)	R_0t	Percent hybridization
cDNA	-	-	5.7
TMV-RNA	75.2	81.2	2.1[a]
Yeast RNA	400.0	432.0	0[a]
WMV - 1 RNA	4.0	4.3	89.1[a] $\pm$ 5.6[b]
WMV - 2 RNA	4.0	4.3	0[a] $\pm$ 0.35[b]

a Hybridization percentages were corrected for self-hybridization of cDNA.

b Standard errors of the mean of three replicates are given for each hybridization reaction.

serologically and biologically distantly related to each other (Greber, 1978; Purcifull & Hiebert, 1979; Makkouk & Lasemann, 1980) were found to have no detectable homology. Therefore, in the BYMV subgroup of the potyvirus group, there is a direct correlation between host range differences with the RNA homology as determined by MHA. The greater the differences in host range, the less homology was observed as seen with WMV-1 and WMV-2 (Greber, 1978).

In virus research, MHA seems to have a place with the large groups of plant viruses such as the tobamoviruses. With small groups of viruses, MHA may be of little value because other properties such as serology are probably sufficient for identification and characterization. Nevertheless, the application of MHA may show differences not detectable by other properties.

The applicability of MHA to new virus systems needs evaluation before they can be adopted for use in identification and characterization of viruses.

ACKNOWLEDGEMENTS

I thank Dr J.W. Randles for helpful discussion and for the facilities provided by Waite Agricultural Research Institute, University of Adelaide, South Australia.

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STUDIES ON TWO TYPES OF ISOLATES FROM ASPARAGUS BEAN WITH MOSAIC SYMPTOMS IN THAILAND

T. Senboku,* S. Kiratiya-Angul**,
W. Srithongchai**, K. Kittipakorn**,
M. Prommin** and N. Deema**

*Tropical Agriculture Research Centre, Ministry of Agriculture, Forestry and Fisheries, Tsukuba Science City, Yatabe, Ibaraki, 305 Japan

**Plant Virus Disease Branch, Plant Pathology and Microbiology Division, Department of Agriculture, Bangkhen, Bangkok 9, Thailand

INTRODUCTION

The occurrence of soybean, mungbean and asparagus bean virus diseases in Thailand was reported by Deema et al. (1976), but no details about the properties of the causal agent were published.

Studies on rice and legume virus diseases occurring in tropical areas, especially in Thailand have been carried out since 1978, in collaboration with scientists from the Tropical Agriculture Research Center, Japan and from the Department of Agriculture of Thailand.

Survey trips to major legume growing areas in northern and northeastern Thailand were undertaken to observe and collect plant samples showing virus disease symtpoms.

The collected plant samples were checked by electron microscopic observations and sap transmission tests.

This paper presents the results of host range tests by sap transmission, electron microscopic observations and aphid transmission tests (by Aphis craccivora) for Asp-SM and Asp-MM, and results of attempts to purify the causal agent of Asp-SM.

MATERIALS AND METHODS

Virus Source:

Diseased asparagus bean samples showing mosaic and vein banding

symptoms were collected from farms in northern Thailand. Attempts were made to maintain these samples by sap inoculation or grafting and parts of the samples were also stored in a deep freezer.

Transmission:

A) Mechanical transmission: sap inoculation was attempted by rubbing sap from leaves prepared in 0.1 M phosphate buffer, pH 7.0 onto carborundum (600n mesh) dusted leaves of healthy test plants.

B) Aphid transmission: virus free Aphis Craccivora Koch were reared on healthy asparagus bean plants. They were given one day acquisition period on diseased asparagus bean plants. The viruliferous aphids were transferred to healthy test plants (seedlings of asparagus bean), and permitted a 2-day inoculation period.

Observation of Virus Particles and Inclusion Bodies

Electron microscopic observations using a Hitachi H300 electron microscope were carried out to detect virus particles. Samples for electron microscopy were prepared for the dipping method by placing carbon-stabilized, formvar-membrane grids on small drops of 2% potassium phosphotungstate pH 7.0, containing chopped leaf tissue pieces from diseased plants. Infected asparagus bean leaves with Asp-SM and MM isolates were fixed with 4% glutaraldehyde in 0.01M phosphate buffer solution (pH 7.2) for 3 days at a temperature of 4°C and post-fixed with 1% osmium tetraoxide for 3 hours and washed with the buffer. After dehydration in an ethyl alcohol series, they were embedded in Spurr's resin. The fixed samples were sectioned with a SORVALL MT 2-13 ultramicrotome with glass knives. The sections were double stained in 2% aqueous uranyl acetate and lead citrate, and examined under an electron microscope (Hitachi H300).

Host range Tests:

At first, the isolation from the samples collected initially was carried out in applying the serial single lesion transfer method. After isolation was completed host range tests were performed. Various test plant species maintained in a screen cage (Table 1), were mechanically inoculated with sap from isolated samples and observed. Symptomless plants were assayed by back inoculation to indicator plant (Mungbean for Asp-SM, Chenopodium amaranticolor for Asp-SM, respectively). All test plants were grown in separate screen cages with insecticides sprayed routinely.

Purification of Asp-sm virus:

Attempts to purify the causal agent of Asp-SM were carried out by extracting sap from frozen asparagus bean tissues which had been collected and stored in a deep freezer 2 weeks after the inoculation. Sap extract was obstained by squeezing the tissues after maceration and homogenization in a 0.5 M phosphate buffer solution, pH 7.5, containing 1%, 2-mercapto-ethanol (ME), 0.025 M ethylene diamine tetra acetate (EDTA), and 0.01 M thioglycolia acid (2 ml buffer per g tissue). The sap was clarified by addition of 0.3 ml chloroform and carbon tetrachloride mixture (1:1, V:VO to 1 ml sap and centrifugation at low speed (3,500 g) for 15 mins. The acqueous phase was recovered, and mixed with 5% polyethylene glycol (# 6,000) including 0.3 M sodium chloride. After one hour of stirring, the mixture was incubated for one hour under cool conditions (about 5°C). The mixture was subjected to centrifugation at 8,000 g for 30 min. Pellets were resuspended in 0.05 M phosphate buffer, pH 8, containing 0.1% ME and 0.005 M EDTA. After homogenization, the resuspended pellets were clarified by addition of 10% volume carbon

Table 1. Host range of Asp-SM and MM.

Plants tested		Asp-SM		Asp-MM	
Family	Species	Inoculated leaves	Upper leaves	Inoculated leaves	Upper leaves
Amaranthaceae	Gomphrena globosa	-	-	-	-
Chenopodiaceae	Chenopodium album	Ch.LL(N.LL)	-	-	-
	C. amaranticolor	Ch.LL(N.LL)	-	Ch.LL	-
	C. quinoa	Ch.LL(N.LL)	-	Ch.LL(N.LL)	-
Cucurbitae	Cucumis sativus	-	-		
Solanaceae	Datura metel	-	-	-	-
	Nicotiana glutinosa	-	-	-	SL
	N. tabacum	-	-	-	-
Leguminosae	Arachis hypogaea	-	SL	-	-
	Cassia tora	-	-	-	-
	Dolichos lablab	-	-	-	mM
	Glycine max (SJ 4)	-	SL(mM)	-	-
	(Tsurunoko)	-	SL(nM)	-	-
	Phaseolus vulgaris (Top crop)	-	SL(mM)	-	SL(mM)
	(Tsurunashi Kintoki)	-	-	-	M
	Pisum sativum	-	-	-	SL
	Vicia fava	N	N,M	N	N,M
	Vigna mungo	-	mM	VN	M
	V. radiata	-	M	N-LL	mM
	V. sesquipedalis	SL	M	SL	mM
	V. sinensis (Black eye)	-	M	-	mM

Key to symptoms:

SL: symptomless infection
Ch: chlorosis
N : necrosis
VC: vein clearing
M : mosaic
mM : mild mosaic
Ch.LL: chlorotic local lesion
N.LL : necrotic local lesion
- : no infection

tetrachloride to the suspension and low centrifugation. The supernatant was broken by one cycle of differential centrifugation (high speed, at 10,000 g, for 90 min; low speed, at 8,000 g for 10 min). The resulting supernatant fluid was layered on 10-40% linear sucrose density gradients, and centrifuged for 2 hours at 25,000 rpm using a Hitachi RPS 25 swing-bucket rotor. After centrifugation, the gradient columns were scanned at 254 nm and fractionated with ISCO density gradient fractionators. Ultra-violet absorbing zone was collected, pooled and concentrated by high speed centrifugation (Fig. 1).

RESULTS

1) Studies on two kinds of isolates from asparagus bean with mosaic symptoms (Asp-SM, MM).

Some diseased asparagus bean samples showing mosaic and yellow banding symptoms were inoculated to healthy seedlings of asparagus bean, kidney bean, mungbean, cowpea, soybean, Chenopodium amaranticolor, Nicotiana glutinosa and petunia, along with graft attempts, for the maintenance of the samples and detection of test plant with local lesions. In some samples, the mungbean tested showed necrotic local lesions on the inoculated leaves, 2-3 days after inoculation. In other samples, Chenopodium amaranticolor was the host with local lesions, showing chlorotic local lesions 5-7 days after inoculation, which subsequently changed to necrotic lesions on the inoculated leaves.

Inoculated mungbean and Chenopodium amaranticolor plants showing local lesions were subjected to isolation experiments in applying the serial single lesion transfer method. Following four or five serial single lesion transfers, two kinds of mosaic symptoms on asparagus bean leaves were observed and isolated. The first consisted of severe mosaic

Fig. 1. Asp-SM Virus Purification

Frozen tissue (diseased Asparagus bean) 150 g

0.5 M pB pH 7.5 1% ME, 0.025 M EDTA, 0.01 M TGA

Maceration, Squeezing

Extract

25% Chloroform and CCl_4 (1:1) Treatment

3,599 g 15 min

PEG Treatment 5% PEG (0.3 M NaCl)

1 hr stirring (in ice bath), 1 hr incubation)

8,000 g 30 min

ppt.

res. 0.05 M Pb pH 8 0.1% ME, 0.005 M EDTA

Homogenization

10% CCl_4 Treatment

Differential centrifugation

100,000 g 1.5 hr

8,000 g 10 min

SDG (10-40%)

25,000 rpm 2 hr

ISCO Scanning

Virus Frac.

symptoms and the other of mild mosaic (tentatively called, Asp-SM and Asp-MM).

2) Host range and symptoms of Asp-Sm and MM.

For Asp-SM and Asp-MM, host range tests were carried out by mechanical sap inoculation to test plants and back inoculation from the tested plants.

The results are shown in Table 2. Sap of asparagus bean leaves infected with Asp-SM and MM, respectively, was inoculated to 19 plant species belonging to 5 families.

A) Asp-SM

Sap of asparagus bean leaves with Asp-SM induced systemic infection in 12 plant species. V. sinensis showed systemic mosaic symptoms, and Chenopodium album, C. amaranticolor, and C. quinoa showed local symptoms on the inoculated leaves. Arachis hypogaea, Glycine max and Phaseolus vulgaris were infected but did not show any symptom.

B) Asp-MM

Sap of asparagus bean leaves with Asp-MM induced systemic infection in 6 plant species which showed mosaic symptoms, namely, Dolichos lablab, Phaseolus vulgaris (Tsurunashi Kintoki), Vicia fava, Vigna mungo, V. sesquipedalis and V. sinensis. Chenopodium quinoa showed local lesions on the inoculated leaves and chlorosis on the uninoculated upper leaves. Vigna radiata showed clear necrotic spots on the inoculated leaves, and subsequently mosaic symptoms appeared on the upper leaves. Chenopodium amaranticolor infected with Asp-MM showed chlorotic or necrotic local lesions only on the inoculated leaves.

3) Comparison between reactions of some breeding lines of asparagus bean to infection with Asp-SM and MM.

Table 2. Reaction of some lines of asparagus bean to Asp-SM and MM.

	Line	Kancha-naburi	K2.1A-72	K2.1A-60	BB.PT-11	K2.1B	75 cen-timeter (Taiwan)
Asp-SM	Middle leaves	+++	+	++	+++	+++	+
	Upper leaves	+	+	+++	+	+	++
Asp-MM	Middle leaves	+	+	++	+	+	+
	Upper leaves	±	±	±	±	±	±

Relative severity of the symptoms
(severe) (mild)
+++ > ++ > + > ±

Asparagus bean plants infected with Asp-SM showed severe mosaic, and yellow banding symptoms as well as malformations. On the other hand, asparagus bean plants inoculated with Asp-MM, showed very fine and mild mosaic symptoms.

Six breeding lines of asparagus bean supplied by the Division of Horticulture, Department of Agriculture, Thailand, were compared for their reactions to infection with Asp-SM and MM.

The results in Table 3 show that no remarkable differences were observed among these lines.

In both samples (Asp-SM and MM), filamentous particles of about 750 nm could be detected.

With regard to the Asp-MM sample, since it was difficult to detect virus particles in the infected asparagus bean, the local lesions on the inoculated mungbean leaves were sometimes used for virus detection.

For Asp-SM and MM isolates, pinwheel or cylindrical inclusion bodies were observed.

5) Aphid transmission of Asp-SM and MM

The results of preliminary tests for aphid transmission are shown in Table 3.

In the transmission tests by aphid, one aphid per plant was used and 20 plants were tested for Asp-SM and MM, respectively. Asp-SM and MM were easily transmitted by Aphis craccivora, with a ratio of active transmission of 40%.

6) Purification of Asp-Sm virus

The opaque band corresponding to the zone containing the virus was recovered from sucrose density gradients. Scanning pattern with ultra-violet light (254 nm absorbance zone) showed the presence of a

Table 3. Aphid transmission of Asp-SM and MM.

	No. of inoculated plants	No. of infected plants	Active transmission ratio (%)
Asp-SM	20	8	40
Asp-MM	20	8	40

Test plant: Asparagus bean
Aphid : Aphis craccivora (one aphid/plant)

peak. However, the preparation showed an ultra-violet light absorption spectrum typical of that of nucleoprotein components with a distinct peak.

7) Seed transmission of Asp-SM

For Asp-SM isolation, seed transmissibility was tested. Four out of 93 seedlings grown from asparagus bean plants infected with Asp-SM isolate were found to be infected with the virus.

DISCUSSION AND CONCLUSION

Two kinds of isolates (Asp-SM and Asp-MM) were recovered from leaves of asparagus bean with severe and mild mosaic symptoms. Final identification of the causal agent is underway.

The isolates showed some similarities with regard to the particle shape of the virus detected by electron microscopy as well as mechanical and aphid transmissibility.

The causal agent of Asp-SM was considered to be the cowpea aphid-borne mosaic virus (CAMV) based on the host range, symptomatology, aphid transmissibility and particle structure.

CAMV belongs to the poty virus group (Bock & Conti, 1974). Until now, four strains of CAMV have been described as follows: the type strain, or European strain, which causes severe distorting mosaic in cowpea (Lovisolo & Conti, 1966); the African strain (called neo-type strain) which resembles the type strain, although infected cowpea cv. Mak 1 shows irregular angular and broken mosaic symptoms (Bock, 1973); the African mild strain which induces very mild mottle; and the African vein banding strain which causes broad dark-green vein banding on cowpea cv. Mak 1, respectively (Bock, 1973).

Jarupat (1975) did not describe the strain reported here in his attempts to identify CAMV in Thailand. Based on the results of the isolation, more detailed observations of symptom expression on cowpea cv. Mak 1 or other test plants are needed for the identification of this particular strain.

Some of the poty viruses cannot be easily purified due to particle aggregation which causes loss of virus yield in the preparation.

Attempts to purify the causal agent of Asp-SM are underway.

Pinwheel and circular inclusion bodies similar to those described in the lesions caused by the poty virus group were observed in the cytoplasm of the infected asparagus bean cells.

The PVY group viruses from Wisteria and Pisum, and the azuki-bean mosaic virus (Hino, 1962) resemble CAMV with regard to some of their properties, and Tsuchizaki et al. (1983) suggested the possibility of the existence of cross-relationships in the serological reaction between CAMV and bean common mosaic virus.

The occurrence of CAMV has been reported in many countries (Hino, 1960; Lovisolo & Conti, 1966; Kaiser et al., 1968; Bock, 1973; Jarupat, 1975) and the wide distribution of CAMV may be related to factors such as transmission, seed transmission and the presence of a wide host range. CAMV is transmitted by Aphis craccivora in a non-persistent manner (Bock, 1973), and Aphis fabae, A. gossypii, A. medicaginis, Macrosiphum euphorbiae and Myzus persicae are known as vectors of CAMV (Vidano & Conti, 1965).

Tsuchizaki et al. (1970) recorded a high ratio of seed transmission (about 20%) of azuki-bean mosaic virus (which resembles the CAMV type strain) in cowpea cv. Kurodane 16.

Therefore, it can be anticipated that CAMV disease will become increasingly widespread in Thailand.

ACKNOWLEDGEMENTS

The authors wish to express their grateful thanks to Miss A. Chandrasrikul, Director of the Plant Pathology and Microbiology Division, Mr P. Surin, Chief of the Oil Crop Pathology Branch, and to the members of the research staff of the Plant Pathology and Microbiology Division for their valuable cooperation.

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YAM BEAN MOSAIC VIRUS IN THAILAND

D. Choopanya, W. Srithongchai, M. Purinunsri, N. Deema
and A. Chanthrasrikul
Division of Plant Pathology, Department of Agriculture, Bangkhen,
Bangkok 10900, Thailand

INTRODUCTION

Mosaic on yam bean was first reported in Thailand in 1977 (Choopanya, 1978). The infected plants showed yellowing and greening of the first leaves, with subsequent spread to the older ones. The infected leaves were rough, crinkled and reduced in size in severe cases. Generally, plant growth and yield were decreased.

This report is on a study of the host range and purification, serology and electron microscopy of the causal organism of yam bean mosaic disease.

MATERIALS AND METHODS

Virus isolated from infected yam bean leaves was used as inoculum. This was made by grinding young infected leaves in 0.5 ml phosphate buffer pH 7.2. Inoculated test plants were kept in an insect screened house. The plants were observed for 3-5 weeks for symptom development.

Purification of virus from infected host: Infected leaves of host plants were harvested 3-4 weeks after inoculation, frozen and homogenized in 0.05 M potassium phosphate pH 7.0 containing 0.1% 2 mereaptoethanol (ME) and 1 M urea (1 g tissue in 3 ml buffer). The extract was squeezed through cheesecloth and four volumes of it mixed with a 1:1 (v/v) chloroform:butanol mixture and stirred for 30 minutes. This was followed by differential centrifugation before further purification by rate-zonal density-gradient centrifugation (Brakke, 1960). The titre of the purified virus was checked by using a Hitachi Model 200-20 spectrophotometer.

Serology: Antiserum for yam bean mosaic virus was produced by weekly injecting the purified yam bean virus preparation into a rabbit for six weeks. The rabbit was bled one week after the last injection. Antiserum was stored in small vials at -70°C. Serology was studied using the microprecipitin method (Ball, 1974). The infected leaves were also prepared for TEM.

RESULTS AND DISCUSSION

Twenty-four plant species were inoculated and only ten species were infected and developed symptoms. Chenopodium album, C. amaranticolor and C. quinoa showed local lesions. Pachyrhizus erosus, Cassia occidentalis, Clitorea ternatia, Glycine max, Phaseolus vulgaris cv. Tsurunashi Kintoki, Phaseolus vulgaris cv. Top crop and Psophocarpus tetragonolobus produced systemic symptoms.

Dipping preparations from infected plant leaves showed flexuous rod particles. The virus contained many fragmented particles with a wide range in length. Approximately 73% were between 650 and 800 nm.

Ultra-thin sections of infected yam bean cells showed semicircular, circular, platelike and pinwheel structures. These inclusions were characteristic of virus in the potato virus Y group as described by Edwardson (1966). The yam bean mosaic virus under study was identified as a potyvirus.

In the purification of virus from inoculated yam bean leaves, differential centrifugation following chloroform-butanol clarification of extracts gave better results than the polyethylene glycel (PEG) method. The viral spectrum was typical of a nucleoprotein with maximum and minimum absorbances at 260 nm and 245 nm respectively, thus giving a ratio of 1.148.

Serological tests for injection periods of 2-4 weeks and 6-8 weeks gave homologous virus titres of 1.512 and 1.1024 respectively.

The results revealed that yam bean mosaic virus was caused by a flexuous rod virus. The average length of the purified particles was 720 nm which is similar to that obtained by Pagnio & Kuhn (1972).

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CONTROL OF CITRUS GREENING DISEASE IN SWEET ORANGE AND MANDARIN

K. Balaraman

Institute for Citrus Research and Development, Bangalore 560024, India

INTRODUCTION

Greening disease, a mysterious malady involving arrested development of entire trees or part thereof together with a wide range of chlorotic leaf symptoms as well as poor crops of under-sized, usually lop-sided and poorly coloured fruits of inferior quality has been known to affect sweet orange Citrus sinensis (Linn.) Osb. and Mandarin orange C. reticulata Blanco. extensively in India. An intensive survey of sweet orange and Mandarin plantations of South India during 1977-80 revealed widespread disease incidence in different regions. While the disease incidence was found to be 15-38% on sweet orange in Andhra Pradesh, it was 22-46% on Mandarin in Karnataka (Kodagu) and Tamil Nadu (Lower Palnies). When Lafleche & Bove (1970) showed greening to be associated with a procaryotic organism and not a virus as previously suspected, they indicated the probability that a method could be devised to control the disease by means of chemotherapy.

The aim of the present investigation was to devise some control measures for the disease with the help of antibiotics.

MATERIALS AND METHODS

Antibiotic therapy

Sweet orange and Mandarin seedlings inoculated with a severe isolate (G-27) of greening pathogen were used. Two antibiotics, Tetracycline-HCl and Penicillin-G at 250, 500 and 1,000 ppm concentrations were injected continuously for two weeks through "wick feeding" method. In this method, the antibiotic solution could be supplied continuously into the stem from a small bottle by means of a cotton thread (wick). One end of the sufficiently long cotton thread was taken

through the stem by means of a needle in such a way that both ends of the thread could dip into a small bottle which could be kept slightly at an elevated height near the stem. The antibiotics could be replenished in the bottle as required.

RESULTS AND DISCUSSION

Plants treated with tetracycline-HCl started producing symptomless new flushes in 65-80 days after injection. The new leaves were normal in size and regained its lustre. The infected old leaves dropped off. At all concentrations of tetracycline-HCl, recovery of plants were noticed. The experimental plants even after 36 months were perfectly healthy and the symptoms never reappeared. There appeared to be complete recovery of the infected plants after tetracycline-HCl injection. Indexing tests from the recovered plants revealed the complete absence of the disease.

Penicillin-G treated plants took 90-105 days to show the recovery from greening. At 250 and 500 ppm recovery was not complete. The immediate new flush was symptomless, but after 4-6 months regained greening symptoms. Plants treated with a higher concentration showed recovery but disease symptoms reappeared in 12-24 months and the greening symptoms could be seen on indexed plants.

In another experiment, the same treatments were given on sweet orange and Mandarin plants budded on rough lemon rootstock. Tetracycline-HCl greatly improved the growth of greening-infected plants within three months, and it was strongly evident 12 months after treatment. In this experiment, penicillin-G at 1,000 ppm alone was tried and remission of symptoms started in 4 months but reappeared in 7 months. Greening reappeared on penicillin-G treated budlings.

The present work suggests that greening-affected plants treated with tetracycline-HCl completely recovered and grew better by producing

normal shoots than untreated controls. Similar beneficial effect was noted by Moll et al. (1980) with tetracycline-HCl treatment on mature plantations in South Africa. However, the results are clearly in disagreement with earlier workers' findings of only temporary remission of symptoms with tetracycline-HCl antibiotics (Capoor & Thirumalachar, 1973; Schwarz et al., 1974; Nariani et al., 1975; Nariani & Bhagabathi, 1980).

Penicillin-G treated plants showed only temporary remission of symptoms, though the concentration and method of application were similar to those using tetracycline-HCl. These results are in agreement with the report that penicillin-G treatment of clover club leaf plants, similar to greening pathogen resulted in symptoms remission (Windsor & Black, 1973). Similarly, Bove et al. (1980) also found that in penicillin-G treatment, the leaf symptoms of greening began to reappear. However, Auburt & Bove (1980), reported that penicillin-G treatment (6 g/tree) induced the formation of new, vigorous, symptomless growth and significant yield increase with quality fruits. Perhaps a higher concentration beyond 1,000 ppm and repeated application may have beneficial effect.

On the basis of the results that the greening organism is sensitive to both tetracycline-HCl and penicillin-G, it is tentatively concluded that Citrus greening is not due to mycoplasma, but a procaryote probably having a peptidoglycan-containing gram-negative type of cell layer.

In a third experiment, field trials laid out during 1981-82 with 24 plants each of sweet orange (C. sinensis) and Mandarin (C. resiculata) budded on Rangpur lime (C. limonia) which were affected with greening naturally, were treated with tetracycline-HCl 1,000 ppm through "trunk

injection" method. The preliminary results revealed the recovery of symptoms in 3-4 months period.

CONCLUSIONS

Both tetracycline-HCl and penicillin-G are effective against greening pathogen. However, tetracycline-HCl can effect complete cure at 250, 500 and 1,000 ppm concentrations for more than 36 months under observation both on sweet orange and Mandarin seedlings and budlings under glass house conditions. A single application of wick feeding for two weeks has a lasting effect with tetracycline-HCl than with penicillin-G. No phytotoxic effect was noticed and indexing of treated plants showed absence of greening pathogen in the system. Preliminary results from field trials on sweet orange and Mandarin confirmed the above findings.

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INFECTION OF *CITRUS* CULTIVARS BY LEAF MOTTLING DISEASE

C.I. Gonzales

Citrus Research and Development, Bureau of Plant Industry,

Lipa City, Philippines

INTRODUCTION

The Philippine *Citrus* is infected by leaf mottling which is both a bud and insect transmitted disease (Salibe & Cortez, 1968; Gonzales *et al*, 1969).

Recent studies indicate that the cause of leaf mottling is a mycoplasma-like organism and not a virus (Lafleche & Bove, 1970; Gonzales & Vinas, 1981; Gumpf, 1981). The insect vector was confirmed to be *Citrus* psylla - *Diaphorina citri*, a jumping plant lice which has no other host plant in the Philippines (Catling, 1968; Altamirano & Gonzales, 1975; Cortez *et al*, 1978).

The study of *Citrus* leaf mottling in the Philippines was carried out at two sites: at the Bureau of Plant Industry Citrus Station (Lipa City), a severely infected area, from 1971 to 1974; and at a mildly infected area in the highland from 1979 to 1982.

In both sites, 4 to 7 year old plantings of *C. reticulata* (Szinkom and Ladu type mandarins), *C. grandis* (pummelo), *C. aurantifolia* (lime), *C. madurensis* (calamansi), *C. limonia* (lemon) and *C. sinensis* Os. (Valencia sweet orange) were marked for data gathering. All plantings were budded on calamandarin stock tolerant to tristeza.

RESULTS AND DISCUSSION

Infection of *Citrus* cultivars by leaf mottling disease

At initial stage of infection, trees of *C. reticulata* (Szinkom and Ladu varieties) developed mottle leaves in one or few branches similar to that caused by mineral deficiency. In most cases, the disease appeared

localised on the tree. The Ladu cultivar tend to show yellow-mottled leaves similar to iron and boron deficiencies. C. madurensis showed dull yellowish green leaves. C. sinensis showed leaf symptoms similar to mild manganese deficiency and localised yellowing in one or few branches. In the case of C. grandis only dull yellowing of leaves was observed. Both C. aurantifolia and C. limonia did not show marked symptoms except for slight yellowing of leaves.

Four years after the initial observation, the marked trees clearly showed progress of the disease. Both cultivars of C. reticulata suffered from dieback of branches and unproductivity. The mottling symptoms similar to mineral deficiency were pronounced and extensive, and the leaves became reduced in size and grew in an upright position. In the case of C. sinensis, leathery and yellowish leaves, premature leaf-drop, dieback of twigs, undersized fruits that fell before maturity and browning of the fruit stem end were characteristic of severely infected trees. Some of the fruits became a-corn shaped and showed inverse colouration (stylar and greening). C. grandis did not show stunting and mottling, but slight reduction in vegetative growth and leaf yellowing. C. madurensis, C. limonia and C. aurantifolia remained without conspicuous symptoms. These cultivars seemed hardy in the presence of the disease but many of the trees showed varying degrees of stem pitting due to natural infection by tristeza virus.

In the mildly infected areas, progress of the disease was quite slow. In the case of C. reticulata and C. sinensis, the symptoms did not multiply rapidly. The trees to a certain extent remained economically productive. The other species - C. grandis, C. madurensis, C. aurantifolia and C. limonia remained mildly infected. It was also noted

that tristeza virus existed in all trees, especially in the susceptible C. madurensis, C. aurantifolia and C. limonia which showed varying degrees of stem pitting.

Etiology of leaf mottling disease

Propagation of severely infected Citrus cultivars using healthy rootstock seedlings gave both disease-free and infected scion growth. The normal growing scions were free from the pathogen. This indicated that the pathogen was either not evenly distributed in an infected tree or present in all the buds (Salibe & Cortez, 1968; Gonzales et al, 1969; Martinez & Wallace, 1969). In mildly infected trees, the pathogen seemed to be confined to the twig or branch showing the symptoms. The normal branch with healthy leaves were disease-free as was shown by the negative results obtained in several attempts to isolate the pathogen from them (Altamirano & Gonzales, 1975; Cortez et al, 1978).

This study reveals that the extent of the disease depends largely on the age of the tree at the time of infection and the high population of its insect vector in the area. The infection seems to depend on external spread through frequent introduction of the pathogen into healthy branches by multiple feeding of the vector Diaphorina citri. It appears that if the pathogen is introduced into a branch of an old tree, it tends to remain confined to that branch for some time without being translocated to the other branches or buds. This was illustrated by artificially infecting single branches of orchard trees. Severely infected trees with complete mottling and yellowing either became infected at the time of propagation or through exposure to frequent feeding of high populations of the vector after transplantation in the orchard.

This study shows that seedlings of many known Citrus cultivarstested by artificial inoculation were infected by the leaf mottling disease. However, different varieties of Citrus differed in their response to the disease (Salibe & Cortez, 1968; Gonzales & Vinas, 1981). It appeared that the Citrus tristeza virus and the leaf mottling mycoplasma-like organism neither acted synergistically on the Citrus cultivars or protect the trees from one or the other pathogen.

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CITRUS TRISTEZA VIRUS CONTROL THROUGH CROSS PROTECTION

K. Balaraman

Institute for Citrus Research and Development, Bangalore 560024, India

INTRODUCTION

Indian citriculture is faced with two major virus or virus-like disease problems. They are (i) Citrus tristeza virus and (ii) greening pathogen. These two diseases affect a wide range of Citrus varieties and create problems generally referred to as "dieback" of Citrus. The area and production have been considerably reduced due to the devastation caused by both maladies. Among the three important species of Citrus grown in India, Mandarin (Citrus reticulata Blanco.), sweet orange (C. sinensis) and acid lime (C. aurantifolia Christm. Swing), the lime is highly susceptible to Citrus tristeza virus (CTV), while Mandarin and sweet orange are sensitive to greening.

CTV is a major disease of acid lime which is mostly grown in South India. It has defied all attempts of control so far. However, recent investigations revealed that mild strain protection (cross protection) phenomenon could be utilized for building up a future lime industry (Balaraman & Ramakrishnan, 1977; 1980). This paper reports the high protection offered by a very mild strain (S_1) under field conditions over a period of 6 years, against the severe (S_5) inoculated strain.

MATERIALS AND METHODS

The experiment was conducted during August 1977, with four treatments: (i) uninoculated acid lime seedlings; (ii) seedlings inoculated with the mild strain (S_1); (iii) seedlings inoculated with the severe strain (S_5) and (iv) seedlings preimmunized with the mild strain and after 8 weeks, super-inoculated with the severe strain (S_1 + S_5). Inoculation was done using the bark patch grafting technique.

RESULTS AND DISCUSSION

The results are presented in Table 1. The mild strain (S_1) inoculated plants grew more vigorously than uninoculated plants. The fruit yield started in a 30-month period and in the 6th year. The trees recorded 3.6 m in height and covered a surface area of 3 m in diameter. The volume of the tree was around 12.6 m^3. On an average the yield was 2,340 fruits/tree/year in its 6th year. This is a 30-fold increase when compared to plants inoculated with the severe strain (81 fruits/tree/year). The uninoculated seedling trees contributed 2,180 fruits, while the cross protected (S_1 + S_5) plants yielded on an average 2,310 fruits/tree.

The preimmunized and cross protected trees had remained stable and with good vigour, although the existing severe strain and high aphid activity were noticed. In the 6th year, 6 out of 36 healthy plants were affected with severe tristeza virus and are still declining.

Citrus tristeza virus disease of acid lime has been of importance to South India. The distribution of the efficient aphid vector (Toxoptera citricida) all over the country and the unrestricted exchange of plants and bud sticks allow the eventual spread of the virus everywhere.

Mild strain protection work initiated earlier at one centre has since been extended to other lime growing regions and the protective interference data shown in this paper appears promising.

The fact that the control plants were naturally infected with severe strains provide evidence that the mild strain inoculated plants are constantly challenged with severe strains under field conditions. Similar location trials on mild stem pitting virus on grapefruit for

Table 1. Growth and yield records of acid lime seedlings inoculated with strains of Citrus Tristeza Virus (CTV).

Treatments	Biometric data			
	Height (m)	Circumference (cm)	Volume (m^3)	Yield No.
1. Uninoculated	3.45	46.5	11.9	2,180
2. Mild (S_1)	3.52	49.0	12.6	2,340
3. Severe (S_5)	1.28	19.0	1.8	81
4. Mild + Severe (S_1 + S_5)	3.49	50.0	12.4	2,310

nearly 33 years in Australia (Fraser & Broadbent, 1979) confirms the present results. Costa & Müller (1980) from Brazil reported good performance of cross protected galego lime and pera sweet orange for over 15 years with 6 mild isolates out of 45 tested. More than 8 million preimmunized plants have so far been supplied to growers in Brazil.

The best performance of cross protected plants reported in this paper from various locations created a demand for propagating materials and already 360,000 plants have been distributed covering an area of 2,600 acres in South India.

Any concern with regard to the strain composition of the CTV, because of deliberate introduction of the mild strain to various regions should be dispeled since repeated sub-cultures of the mild strain (nearly 30 times until 1982) did not show any change in its behaviour. Furthermore, the field trees, even after 6 years of exposure do not show any breakdown in protection. It is hoped that the high or complete protection offered by the mild strain be a permanent future and repeated inoculations need not be repeated.

The mild strain protection phenomenon has since been extended to lemon, sweet orange, Mandarin, pomelo, grapefruit and other species and varieties of Citrus under field conditions. Preliminary results indicate high protection offered all the species of Citrus.

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CITRUS TRISTEZA VIRUS IN THAILAND AND THE PRODUCTION OF VIRUS FREE PLANTS

S. Attathom, S. Bureekum, Y. Monkolasuk, S. Bhongsatien and K. Rienwarakorn

Department of Plant Pathology, Kasetsart University, Kamphaengsaen Campus, Nakorn Pathom. 73140, Thailand

INTRODUCTION

Citrus plants grow well in Thailand. Commercial varieties are Citrus reticulata Bl. (som-khiewwan), C. sinensis Osb. (som-tra), C. aurantifolia Swingles (manao) and C. grandis Osb. (som-o). The limiting factor in Citrus production is plant diseases, many of which the causal agents are still not known. The economic importance of Citrus tristeza virus disease (CTV) to Thai Citrus industry is not well recognized by Citrus growers because of the lack of conclusive information on the etiology of the disease. The detection of CTV in commercial Citrus varieties by rapid diagnostic methods and its possible control are reported here.

MATERIALS AND METHODS

Diagnosis of Citrus tristeza disease: Immuno electron microscopy (IEM) (Derrick, 1973) using CTV antiserum (Tsuchizaki et al., 1978) and enzyme-linked immunosorbent assay (ELISA) (Clark & Adamns, 1977; Bar-Joseph et al., 1980) were used for CTV detection.

Ultrastructure of CTV infected cells: Tissues 1 x 3 mm in size were collected from midveins of CTV infected plants. The methods of Kitajima & Costa (1968) and Kitajima et al., (1974) were used to prepare the specimens for IEM.

Production of virus-free plant: Shoot-tip grafting in vitro as described by Navarro et al., (1975, 1976) was used to produce CTV-free

Citrus plants. Rootstock seedlings were obtained from Citrus seeds cultured in Murashige & Skoog medium (Murashige & Skoog, 1962). Shoot-tips were excised from field grown C. aurantifolia and C. grandis or from shoots derived from lateral buds cultured on Murashige & Skoog medium. Successfully grafted plants were kept for 2 weeks in test tubes before transplanting. They were also assayed for CTV by IEM.

RESULTS

Host range and symptomatology: Infected C. aurantifolia (manao) plants showed typical vein clearing, dieback of young twigs, leaf thickening and malformation. The so-called "quick decline" symptoms consisting of leaf and fruit droppings, wilting and death of plants were also observed. Expression of CTV symptoms in C. reticulata (som-khiewwan) in field conditions was related to plant age. In 1-5 year old orchards, infected plants showed stunting and yellowing. Decline symptoms consisting of leaf chlorosis, leaf and fruit droppings, dieback and death were observed in older plants. Infected C. sinensis (som-tra) showed severe leaf chlorosis and mottling, leaf and fruit size reduction and dieback, symptoms resembling greening disease. New shoots might develop without noticeable symptoms. External symptoms were not pronounced in CTV-infected C. grandis (pomelo) plants except in a few cases where leaf mottling and corky veins were observed.

CTV-diagnosis: CTV is identified by its long flexuous rod of 1,000-2,000 nm in length. IEM: Derrick (1973) gave satisfactory results for CTV detection, especially for C. aurantifolia (manao) and C. reticulata (som-khiewwan), and was routinely used for Citrus tristeza virus diagnosis. ELISA test worked well with extracts obtained from infected plants which gave an easily visualized yellow colour. When it

was used to estimate the concentration of CTV in leaf tissue, leaf petiole, and bark of C. reticulata (som-khiewwan), the results showed that the highest CTV concentration was in leaf petiole (Table 1).

The presence of CTV in infected Citrus plants confirmed the etiology of the disease. Infected cells revealed flexuous rods forming bundles, or were fully packed in phloem cells. Membrane bounded vesicles were observed in vascular tissues of infected plants.

Production of CTV-free plant: Shoot-tip grafting in vitro was useful for the production of CTV-free C. aurantifolia (manao) and C. grandis (pomelo) plants. The percentage of successful grafts was low with 30-40% average for C. aurantifolia (manao) and 10% for C. grandis. Shoot-tip grafted plants were subjected to IEM for CTV detection. The results were all negative, indicating that they were CTV-free.

DISCUSSION

Our work demonstrated the presence of CTV in all commercial Citrus varieties in Thailand. The so-called "quick decline" symptoms of C. aurantifolia (manao) and C. reticulata (som-khiewwan) were due to CTV which is especially destructive of C. reticulata (som-khiewwan) and C. aurantifolia (manao). The effects of CTV on other Citrus varieties are rather difficult to ascertain as most of the severely infected trees are frequently replaced by new plants.

CTV-free plants provide an alternative approach for Citrus tristeza virus disease control in Thailand. To start a national programme for disease-free plants, effective control of the insect vector (oriental Citrus aphid) should be developed. Research should also focus on different CTV strains on Citrus plants so that growers can be alerted on any outbreak of a severe strain. Pre-immunization of CTV-free plants

Table 1. Comparison of ELISA values (OD_{405}) of extracts of leaf tissue, leaf petiole and bark of CTV infected C. reticulata (som-khiewwan).

Sample No.	Leaf tissue	Leaf petiole	Bark
1	0.893*	1.714	1.240
2	0.634	1.306	1.365
3	0.769	2.000	0.473
4	1.476	1.926	1.451
5	1.826	1.932	1.971
6	1.625	1.766	2.000
7	2.000	2.000	2.000
8	1.459	2.000	0.988
9	1.497	1.784	1.807
10	0.664	1.288	2.000
11	1.968	2.000	1.873
12	1.573	2.000	1.706
13	1.408	1.714	0.290
14	1.638	2.000	0.339
15	1.057	2.000	0.168
16	1.477	2.000	1.155
17	1.218	1.699	0.673
18	0.819	1.888	0.486
19	0.453	2.000	0.991
20	0.319	2.000	1.200
Average	1.238	1.850	1.231

*Average A_{405} values of healthy C. reticulata (som-khiewwan) = 0.293.

should be seriously considered.

ACKNOWLEDGEMENTS

We thank S. Chooprayoon and P. Srifa for their assistance. This research was supported by the National Research Council.

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VIRUS-FREE PLANTLETS PROPAGATED FROM INFECTED MOTHER TISSUE

R. Utayanugol and S. Vora-Urai*

Prince of Sonkla University, Hadyai 90110, Thailand

*Chiang Mai University, 50000, Thailand

INTRODUCTION

Cymbidium mosaic virus (CyMV) is a RNA - containing flexuous rod of ca. 475 x 13 nm, readily transmitted by inoculation of infected plant sap to certain host plants. Its vector is not known. Infected plants normally produce mosaic, necrosis, leaf enation, shoot twist and decrease in number of blossoms. CyMV caused severe damage to orchid flower production of Dendrobium Pompadour. The spread of this virus presumably occurred by mechanical injury from contaminated cutting knives during clonal propagation or by flower cutting. Some special techniques such as heat treatment (Hollings, 1965), the rapid proliferation of callus cells and the aid of tissue culture method may help in obtaining virus-free plants from infected species.

MATERIALS AND METHODS

CyMV infected and healthy mature Dendrobium Pompadour were obtained from orchid growers in Chiang Mai and Bangkok. Seedlings of Cassia occidentalis Linn. and Chenopodium amaranticolor Coste & Reyn were used as test plants. Carborandum with 600 mesh was used as abrasive.

The Cassia and Chenopodium plants of 15 and 30 days old respectively were grown in the glass house. Before inoculation with plant saps, leaves of these test plants were dusted with 600 mesh carborandum.

The inoculum was prepared by macerating one gram of tissue with pestle and mortar in 2 ml of 0.1 M, pH 7.2 phosphate buffer. The crude sap was then finger rubbed on leaves of the test plants previously

prepared for the infectivity test. The leaves were then rinsed with tap water and kept in the glass house.

The excised portion of the shoot apex with 1 to 2 leaf primordia separated from the mother plant, previously proved to be CyMV-infected, was then aseptically transferred into the liquid culture media (Table 1). The formulae used in the experiment were P. Voraurai's formula (personal contact) and the modified formula of Vacin and Went (Tables 2 and 3). When the growth of callus was pronounced, the tissue was cut into 3 or 4 pieces. The pieces of calli were then subcultured onto 3 modified media for about 2 months. The plantlets (1.5 cm height) of the cluster were individually separated and then transferred onto a third modified semi-solid medium for half month and they were then ready for the infectivity test.

DISCUSSION

Lawson & Ali' (1975) found that electron microscopy and infectivity test for the virus gave the same positive result of the CyMVinfected Cattleya in response to sensitivity. They stated that infectivity test could be used by commercial growers because the procedure was simple and the least expensive. For the detection of CyMV in D. Pompadour, infectivity test is a practical one since electron microscopy is not readily available. The CyMV symptoms, can be easily detected on Cassia leaves 3-5 days or 25 days on leaves of Chenopodium after inoculation has been made. From the results of the infectivity test, Dendrobium plants and their mericlones gave brown spot local symptoms on Cassia leaves and large grey spot symptoms on Chenopodiium leaves, thus indicating they were indeed caused by CyMV.

The results of the detection shown in Table 4 indicated the

Table 1. The number of plantlets obtained from some shoot apex culture.

Size of cut shoot apices (mm^3)	No. cut shoot apices per treatment	No. proliferated shoots per treatment ($4^1/2$-5 mth old)	Coded	No. plant-lets per culture
0.5 x 0.5 x 0.3	61	4	D_{86}	23
			D_{98}	11
			D_{62}	1
			C_{11}	11
1.0 x 1.0 x 0.5	67	3	D_{38}	22
			D_{80}	21
			D_{16}	7
2.0 x 2.0 x 1.0	76	4	D_{94}	5
			D_{23}	13
			D_{36}	5
			D_{65}	11

D coded for the shoot apices cut from the new shoots of tested Pompadour from Susan Gurab Chiang Mai.

C coded for apex of Pompadour obtained from Bangkok.

The numerical number below the letter showed the labelling number of tested D. Pompadour.

Table 2. The composition of P. Voraurai's medium.

Major salts	mg/l	Minor salts	mg/l	Organic supplements	mg/l
NH_4NO_3	213.00	$AlCl_3$	0.03	Glycine	2.00
$(NH_4)_2SO_4$	370.00	$CuSO_4.5H_2O$	0.03	Nicotinic acid	0.50
KH_2PO_4	550.00	H_3BO_3	1.00	Pyridoxine.HCl	0.50
KNO_3	1,050.00	KI	0.01	Thiamine.HCl	0.50
$Ca(NO_3)_2.4H_2O$	800.00	$MnSO_4.4H_2O$	0.10	Meso-inositol	100.00
$MgSO_4.7H_2O$	500.00	$NiCl_2.6H_2O$	0.03	IAA	2.50
Na_2EDTA	37.00	$ZnSO_4.7H_2O$	1.00	Kinetin	0.20
$FeSO_4.7H_2O$	27.80			Sucrose	20,000.00

The above composition was diluted with distilled water and the pH of the solution was adjusted to 5.6 $\pm$ 0.05 with 1.0 M NaOH or 1.0 M HCl. The final volume was made up to 1,000 ml and distributed to 125 ml Erlenmeyer flasks, 10 ml each. The flasks were capped with heavy duty aluminium foil and autoclaved for 15 minutes at 15 lb/in^2.

Table 3. Modified composition of Vacin and Went's medium.

Nutrients	mg/l
$Ca_5(PO_4)_3(OH)$	200.00
KNO_3	525.00
KH_2PO_4	250.00
$MgSO_4.7H_2O$	250.00
$(NH_4)_2SO_4$	500.00
$C_6H_5O_7Fe.5H_2O$	28.00
$MnSO_4.2H_2O$	7.50
Sucrose	20,000.00
Agar	5,000.00

The above medium was added with various organic compounds such as 0.6 mg/l NAA, 500 mg/l casein hydrolysate, 500 mg/l bacto malt extract, 1 mg/l thiamine.HCl, 100 mg/l meso-inositol and 20 to 15% coconut milk. The pH of the medium was adjusted to 5.0 $\pm$ 0.05 and the final volume was made up to 1,000 ml. The mixture was boiled to melt the agar and distributed to 125 ml Erlenmeyer flasks or 25 x 250 mm culture tubes, 25 ml each. The culture tubes were capped with cotton wool plugs and sterilized as previously mentioned.

Table 4. The number of virus-free plantlets grown from the small shoot apices of mature plants which showed various degrees of CyMV infection.

Coded	Degrees of CyMV* infection in mature D. Pompadour	Detection of CyMV in plantlets 1st test		2nd test		Total virus-free plantlets after the reconfirmed test
		Positive	Negative	Positive	Negative	
D_{86}	+++	2**	21	0	23	21/23
D_{98}	+	10	1	10	1	1/11
D_{62}	+	0	1	0	1	1/1
D_{11}	++++	0	11	0	11	11/11
D_{38}	+	11	11	12	10	10/22
D_{80}	+++	3	18	3	18	18/21
D_{16}	+	3	4	3	4	4/7
D_{94}	+	4	1	4	1	1/5
D_{23}	+	10	3	12	1	1/13
D_{36}	+	1	4	2	3	3/5
D_{65}	++	6	5	10	1	1/11
				Total		72/130

* = + represented mild infection; ++ represented moderate infection; +++ or ++++ represented severe infection.

** = possibly resulted from accidental error.

mildness and severity of CyMV infection in D. Pompadour plants. The number of spots on the leaves were very varied. This suggested that there were different degrees of virus infection. The first tested plantlets of the mericlones derived from cut apices of about 0.5 x 0.5 x 0.3, 1 x 1 x 0.5 and 2 x 2 x 1.0 mm^3 contained either detectable or non-detectable amount of the virus particles, e.g. clonal plants of D38, D23, D36 and D65 (See Tables 1 and 4) gave ratios of positive test to the total test as 11/22, 10/13, 1/5 and 6/11 respectively. Small plantlets with first negative test were confirmed for the CyMV infection one month later by the same procedure. The increase in the positive test indicated that non-detectable virus, in the first test, can multiply within one month to a detectable amount. 72 out of a total of 130 tested plantlets from the reconfirmed test were still virus-free. 47 of the virus-free plantlets from the second test were kept one more month and proved for the thirdtime, remain virus-free.

Infected plantlets most likely originated from the tissue contain-ing vascular strands which may have been the main passage for virus distribution in the developing tissues. The virus-free plants in the other group were probably derived from the apical meristematic tissue containing no vascular supply. In such a case, the virus may slowly have entered into apical meristematic cells from the vascular strands through plasmodesmata. The meristematic cells could probably escape the infection by means of much more rapid cell division than the multiplica-tion of the virus. Thus new dividing cells may be developed into a new plant body which is free from virus. This similar natural escape from some plant virus infections were reported by Hollings (1965).

The proliferation of D86, C11 and D80 from shoots of severely infected plants produced more virus-free plantlets than D98, D38 and D23 which were derived from shoots of mildly infected plants. This may be interpreted that severely infected plants produce new-born shoots containing less particles since they may grow during the period that the particles dropped in population. On the other hand, virus-infected plantlets may come from shoots of mildly infected plants because the shoots may grow during or after the virus has increased in population. The division of meristematic cells under such condition became unable to escape infection, thus more infected plantlets were produced.

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RECENT DEVELOPMENTS ON THE DETECTION, MOLECULAR STRUCTURE AND VARIATION OF THE COCONUT CADANG-CADANG VIROID

J.S. Imperial

Virology-Biochemistry Division, Philippine Coconut Authority,

Albay Research Center, Guinobatan, Albay 4908, Philippines

DOT HYBRIDIZATION ASSAY

The specific and sensitive assay of the coconut cadang-cadang viroid (CCCV) which is done by molecular hybridization with a radioactive complementary DNA probe, provides unequivocal diagnosis for cadang-cadang infection. Molecular hybridization as a tool for detecting CCCV was originally developed in the liquid medium (Randles & Palukaitis, 1979). More recently, the technique by hybridizing extracts bound to nitrocellulose membrane (dot hybridization assay) (Owens & Diener, 1981) was adopted for CCCV-containing samples using a ^{32}P-labelled nick-translated recombinant DNA probe which contains sequences complementary to CCCV (Symons, unpublished).

In the dot hybridization assay, the samples are spotted on a piece of nitrocellulose membrane sheet, fixed, pretreated, and hybridized with the probe. The CCCV-recombinant cDNA hybrids are then detected by autoradiography. Comparison of the assay results of successive five-fold dilutions of various palm extracts with conventional polyacrylamide gel electrophoresis assay (Randles, 1975) gave a 100-600 fold higher sensitivty for the dot assay. The gel assay which gives reliable results with coconut leaf samples of ten times gives ambiguous gel patterns with leaf extracts from other palms or plants. This ambiguity is resolved with the use of the molecular hybridization assay.

The dot assay has been successfully used with leaf samples of various species of test plants in our host range study. Future

activities which will utilize this technique include identification of insect vectors of cadang-cadang and the very early detection of infection in coconut palms.

MOLECULAR STRUCTURE

The complete nucleotide sequence and secondary structure of CCCV was elucidated (Haseloff et al., 1982) simultaneous with the confirmation of the infectivity of pure preparations of the viroid (Mohamed & Imperial, 1983). The smallest form of CCCV is a single-strand covalently closed circular RNA. It is made up of 246 nucleotide residues and shares 2 regions of sequence homology with the potato spindle tuber viroid (PSTV), the citrus exocortis viroid (CEV), and the chrysanthemum stunt viroid (CSV), each region consisting of around 20 base residues (Haseloff et al., 1982). Two-thirds of the total number of nucleotides are predicted to form intramolecular base pairs which give rise to a linear molecule under non-denaturing conditions. The molecule appears very much like the other viroids due to the presence of short double helices and small internal loops. The linear secondary structure was confirmed in measurements on the hydrodynamic properties of CCCV molecules (Riesner et al., 1982). The rod-like structure of CCCV in the native state was initially observed in electron micrographs (Randles & Hatta, 1979) and measurements on the smallest form under denaturing conditions gave a contour length of 107 nm (Imperial et al., 1981).

CCCV also shares dynamic structural features with the other viroids. Data obtained from denaturing or melting experiments demonstrated a cooperative main transition between 45°C and 49°C which results in the formation of a stable hairpin-containing circle, and a second transition at a slightly higher temperature in which dissociation of the hairpin structure occurs (Randles et al., 1982). Unlike PSTV,

however, which showed a number of stable hairpins, only one was observed for CCCV.

MOLECULAR FORMS

A property that is uniquely well-defined in CCCV is the existence of several forms of the viroid. Three types of variation have been characterized. The first which was already apparent when CCCV was first detected (Randles, 1975) arises from the dimerization of the CCCV monomer (CCCV-1) to form CCCV-2 (Mohamed et al., 1982). Both forms are present in the palm throughout the duration of the disease. The dimer CCCV-2, is also a single strand covalently closed circle in which the intramolecular base pairs in each of the monomeric units are maintained (Haseloff et al., 1982).

The second type of variation arises from the duplication of a nucleotide sequence in the small CCCV-1 (referred to as CCCV-1 to denote fast electrophoretic mobility) to form the larger molecule, CCCV-1 s (s denotes slow electrophoretic mobility). A nucleotide region which varies from 40-55 base residues located at one end of the rod-like CCCV-1 f native molecule is repeated at the same portion of the molecule to form CCCV-1 s. The base pairings in the repeated regions are retained so that the same overall molecular configuration is obtained (Haseloff et al., 1982). Parallel variations are also observed in the dimer, that is, whenever CCCV-1 f or CCCV-1 s is detected the respective dimers are also present (Imperial et al., 1981). In naturally infected trees the fast forms are first observed during infection (Mohamed et al., 1982), and with disease progress these are replaced by the slow forms (Imperial et al., 1981).

The third type of variation results in the existence of subforms

of each of the four species of CCCV which differ only by the presence or absence of the cytosine residue at the position corresponding to residue 198 of CCCV-1 f (Haseloff et al., 1982). The occurrence of these subvariants was observed to depend also on disease development in the palm. Two sequences of appearance have been detected (Imperial & Rodriguez, 1983). In the first sequence $CCCV_{246}$ (CCCV-1 f without 198 cytosine is made up of 246 base residues) first appears in the palm and as disease progresses $CCCV_{247}$ (CCCV-1 f with 198 cytosine) appears which may completely replace $CCCV_{246}$. Shortly, $CCCV_{296}$ (CCCV-1 s without 198 cytosine) appears and the f forms disappear. In the time $CCCV_{297}$ (CCCV-1 s with 198 cytosine) appears which may also completely replace $CCCV_{296}$. In the second sequence $CCCV_{247}$ is first detected and this is replaced by $CCCV_{297}$ without the intermediate appearance of $CCCV_{246}$ and $CCCV_{296}$.

There is no detectable movement of CCCV into the adjacent fronds. Once a young frond opens and is still free of CCCV it remains so despite the presence of CCCV in the next frond. Furthermore, whatever forms are present in the frond while it is still young remains the same till it matures in the crown. These observations indicate that the various mechanisms which produce these specific changes in the viroid molecule functions before the fronds open.

Experimental transmissions into young coconut seedlings demonstrate that both fast and slow forms of CCCV-1 and CCCV-2 are infectious (Mohamed & Imperial, 1983), however, inoculum containing the fast forms was shown to be more infectious than the one containing the slow forms (Imperial et al., 1981). In naturally infected palms, only the fast forms of CCCV are detected first and this can either be $CCCV_{246}$ or $CCCV_{247}$. This suggests that only the fast forms may be involved in the natural spread of the disease.

Infectivity tests that will take at least two years have been started using purified subvariant forms. The occurrence of subvariants other than the ones characterized have been detected in CCCV isolates from various areas within the geographic distribution of the disease. The nature of their occurrence is still being investigated. This further detection of other forms of CCCV in addition to the several forms that have been characterized indicate the complexity of the nature of the pathogen causing cadang-cadang disease of coconuts in the Philippines.

ACKNOWLEDGEMENTS

This review is based on studies carried out with the support of the Philippine Coconut Authority, UNDP-FAO and the University of Adelaide. The author gratefully acknowledges the contributions of numerous researchers from each of these organizations.

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APPENDICES

WELCOME ADDRESSES

Professor A N Rao, Chairman, Organising Committee and Head of Botany Department, National University of Singapore

Professor Bernard Tan, distinguished guests from overseas, participants, dear colleagues, ladies and gentlemen,

It gives me very great pleasure to welcome you all to this morning's Opening Ceremony. For those who have come from overseas we also welcome you to Singapore and our University. We have been working for some months to organise this Workshop on Plant Viruses and Mycoplasmas.

The idea of organising this Workshop came to our minds i.e., Professor G Lim, Dr S P Raychaudhuri and myself when Dr Raychaudhuri came to visit our department a few years back as a guest lecturer. We realised that in the field of plant viruses and mycoplasmas there was very little research done in this part of the world though plenty of scope was available, calling the attention of plant biologists. In a way this is true of certain other areas of tropical Botany or even Biology. Even in teaching, plant viruses are considered very briefly in many of the Asian universities.

It is well known that many biologists in Asia have less opportunity to meet fellow scientists either from the neighbouring countries or in the region and to discuss problems and matters of common interest. Invariably they may be better acquainted with their counterparts in UK, USA or other European countries. To cover some of these deficiencies, such small meetings are very helpful and it will bring better coordination among the researchers in the same field. Organising such small but meaningful meetings has been one of the main

interests of COSTED for some time. Many mycologists and virologists have come to attend this Workshop meeting today.

The objectives of this meeting are very well outlined and I have no doubt that there will be fruitful exchange of many new ideas and information.

For organising any meeting of this nature, there are two essential components: 1) manpower; and 2) finances.

Professor Lim, Dr Tan, Dr Yeoh and my other colleagues have provided the manpower in planning and organising this meeting. Primarily it is their efforts that have resulted in bringing all of us together here this morning.

The second component, namely financial help, is extended by UNESCO and COSTED. Professor S Radhakrishna, Secretary of COSTED i.e., Committee on Science and Technology in Developing Countries, is not with us this morning due to his commitments elsewhere. UNESCO and COSTED have supported many other similar activities in our University before and our working relationships are improving to the extent that many of the programmes and meetings are carried out successfully after just one or two discussions.

One of the objectives of the Workshop is to summarise research, development and technological advances made in plant viruses, mycoplasmas and various plant diseases caused by them, particularly in the Asian region. Plant viruses are interesting whether we consider their pathological, physiological, genetical or biochemical aspects. As early as 1892 the infectiousness of tobacco mosaic disease was shown to be transmitted via cell free filtrates from leaves of disease plants to leaves of healthy plants. They were first called 'filterable virus'

and later the word 'filterable' was dropped. The tobacco mosaic virus was also the first to be crystallised. Subsequent research gave a clear understanding that a virus is not an organism but a combination of nucleic acid and protein. Both genetically and biochemically they are very fascinating since they are the only units of entities in the entire biological world that can remain with or without the 'life factor' at one and the same time. To put it very crudely they are immortal in one sense or even literally if we can use such a word, with the permission of the philosophers.

Comparatively, mycoplasmas are recent entries into the field since they were identified in 1967 by the Japanese workers Doi and others. They are more like true micro-organisms but of very small size and apparently without rigid cell walls.

In this assembly, we have overseas scientists from Brunei, Malaysia, Indonesia, Thailand, Philippines, Japan, India, Taiwan and Singapore, representing nine Asian countries. All of you will be busy for the next few days discussing many interesting details. For our overseas visitors we wish you a very happy and pleasant stay in Singapore. We shall be glad to help and extend our assistance to make you comfortable during your stay here both on and off the campus.

On behalf of the organisers and our department, I extend our grateful thanks to Professor Bernard Tan, Vice-Dean, Faculty of Science for agreeing to inaugurate this Workshop. Our thanks are due to all our colleagues in the department who have worked hard in many ways in the organisation of the Workshop. The financial help given by UNESCO and COSTED is gratefully remembered. In particular we are thankful to Mrs Rudstrom who is in charge of higher education and research at

UNESCO, Paris, Mdm Julia Marton-Lefevre, Secretary, ICSU and Professor Radhakrishna, Secretary, COSTED. The officials at the Singapore Tourist Promotion Board have helped us with invitations and other matters and we thank them for their courtesies.

We are also thankful to all of you for having kindly responded to our invitation and for having graced the occasion with your kind presence.

Now it is my pleasant duty to call upon Professor Bernard Tan to declare the Workshop open.

OPENING ADDRESS

Associate Professor Bernard Tan, Vice-Dean, Faculty of Science,
National University of Singapore

May I first of all express my thanks to the Department of Botany for inviting me to open this Workshop on Plant Viruses and Mycoplasmas today. I note with interest that the Workshop also has the sponsorship of UNESCO and COSTED, which has made possible the attendance of several virologists from countries in this region.

The importance of agriculture to the countries of this region cannot be overstated, in relation both to food production as well as to the general economic well-being of these countries. The economies of many countries in this region are based on agriculture and therefore the well-being of their crop plants to a large extent determines the well-being of their citizens. Unfortunately, like animal life, plants are subject to a wide range of ailments and diseases, which can be caused by a wide variety of agents or pathogens, such as bacteria, fungi, viruses, mycoplasmas and others.

Crop plants are particularly susceptible to viruses and virus-like diseases, and such diseases can spread extremely rapidly, causing grave economic losses to the crop growers and to the country. Such losses can easily run into the millions, thus adding to the problems of market prices and manipulations faced by these countries when they sell their crops in international markets.

Of course scientists can do little about economic recession and market manipulation, but happily, something can be done about the diseases which blight the agricultural produce of these countries. Much work, I understand, has been done on virus diseases of plants in the developed countries, and it is of course hoped that any applicable

results will be made available to the scientists of the developing world where most of the problems with virus diseases of crops are to be found. However, it stands to reason that as much research as is possible should be done in the developing countries themselves, so that the application of the ways and means to combat virus diseases can be more direct and thus more efficient.

I am happy to note that there is indeed an increasing amount of research activity on virus and virus-related diseases of plants being done in developing countries. Such efforts can be made more effective if the scientists in developing countries pool their efforts and develop joint research programmes, or at least exchange results freely among themselves. All too often, scientists in developing countries find it easier to communicate with their counterparts in the developed countries in comparison to those in other developing countries. One way by which they can improve communication among themselves is by regional seminars and conferences such as this one. On the other hand, we still value the cooperation of our colleagues in the developed countries, and we hope that they will continue to freely make their research results available.

In this respect, though Singapore has little or no agriculture of its own, Singapore botanists have a very real stake in participating in research which deals with agricultural and primary produce. We in Singapore, would like to make our contribution to the continuing efforts in this region as well as in the other agricultural regions of the world, to improve the food resources and hence the general quality of life of the peoples of the region. In addition, we hope that Singapore, with relative ease of communication with the rest of the

region, can serve as one of the focal points through which botanists and other scientists can disseminate and discuss their research findings throughout the region.

In conclusion, may I wish that all of you have a fruitful workshop during which you will be able to exchange research results of use with one another. I also hope that those of you from overseas will be able take some time off to see the rest of this University as well as to explore a little of our island republic. I now have great pleasure in declaring open this Workshop on Plant Viruses and Mycoplasmas. Thank you.

ACKNOWLEDGEMENTS

The Organising Committee of the Workshop thanks:

Associate Professor B. Tan, Vice-Dean, Faculty of Science, National University of Singapore for his address and opening of the Workshop;

Professor A.N. Rao, Head, Botany Department for his address and also for officiating on behalf of COSTED at the opening of the Workshop;

Professor Lim Pin, Vice-Chancellor, National University of Singapore for providing University facilities;

UNESCO and COSTED for sponsoring the Workshop;

Dr. S.P. Raychaudhuri, who proposed this idea of a Workshop;

Dr P.C. Leong and Mr T.A. Yong of the Sembawang Field Experimental Station, Ministry of National Development for the Field Trip hospitality;

Mrs. Goh-Sim of the Singapore Tourist Promotion Board for courtesies; Singapore Science Centre, numerous friends, colleagues and assistants who have all helped towards making the Workshop possible; and last but not least, the secretarial staff of the Botany Department, especially Joan Choo and Sujatha Menon, for their assistance in the typing of the manuscript.

LIST OF PARTICIPANTS/OBSERVERS

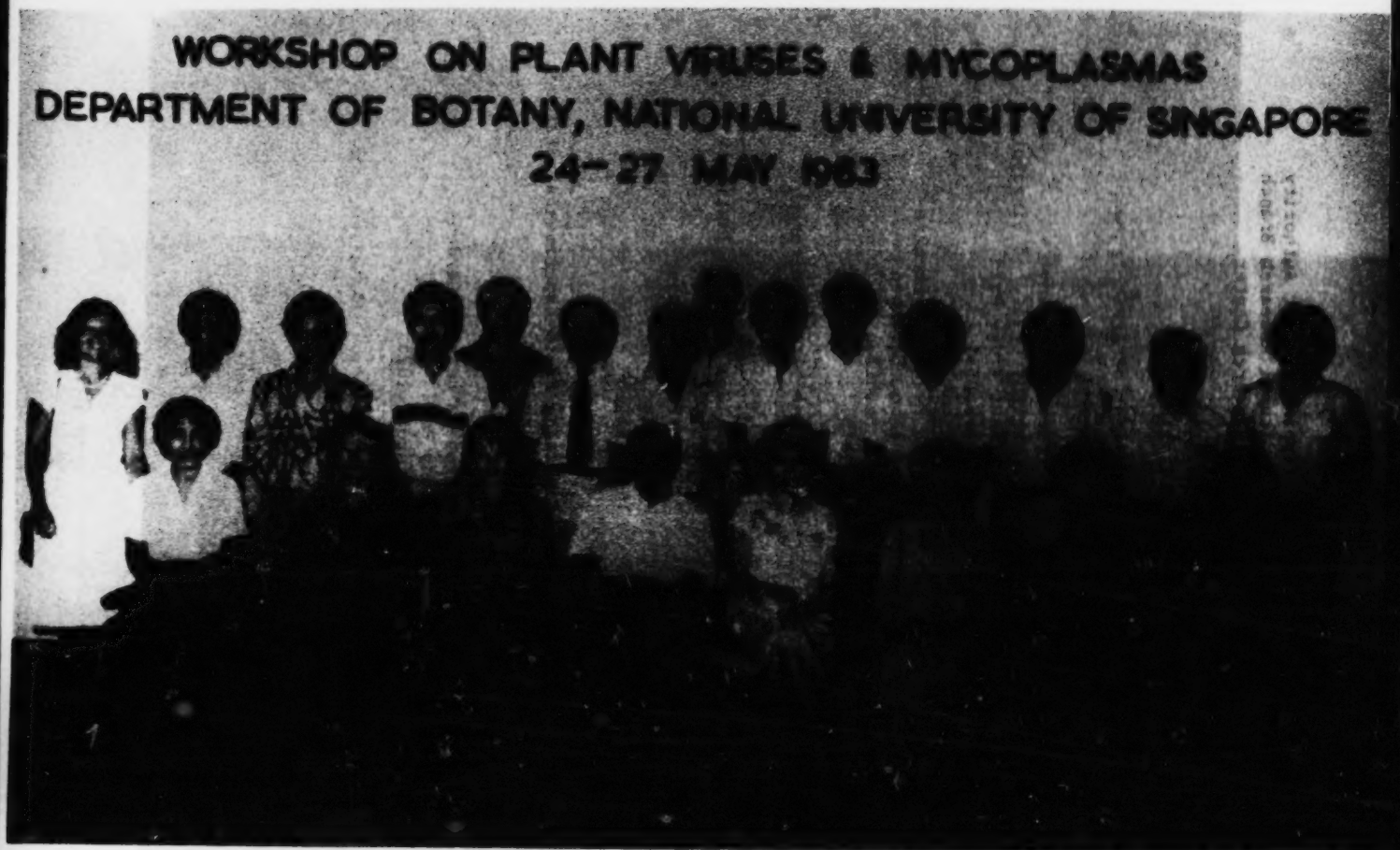
Participants

Dr N Abdul Samad	-	Department of Biochemistry & Microbiology Faculty of Science & Environmental Studies Serdang, Selangor Malaysia
Dr S Attathom	-	Department of Plant Pathology Kasetsart University Kamphaengsaen Campus Nakorn Pathom 73140 Thailand
Dr K Balaraman	-	No. 4, AG's Colony, Hebbal, Bangalore 560024 India
Dr D Choopanya	-	Division of Plant Physiology Department of Agriculture Bangkhen, Bangkok 10900 Thailand
Dr C I Gonzales	-	Bureau of Plant Industry Ministry of Agriculture Lipa City 4216 Philippines
Dr S Green	-	Asian Vegetable Research & Development Center P O Box 42, Shanhua Tainan 741 Taiwan, ROC
Mrs J S Imperial	-	Philippine Coconut Authority Agricultural Research Branch Albay Research Center Banao, Guinobatan Albay 4908 Philippines
Professor G Lim	-	Botany Department National University of Singapore Lower Kent Ridge Road Singapore 0511
Professor A N Rao	-	Botany Department National University of Singapore Lower Kent Ridge Road Singapore 0511
Dr M Raychaudhuri	-	Indian National Science Academy Bahadur Shah Zafar Marg New Delhi 110 002, India

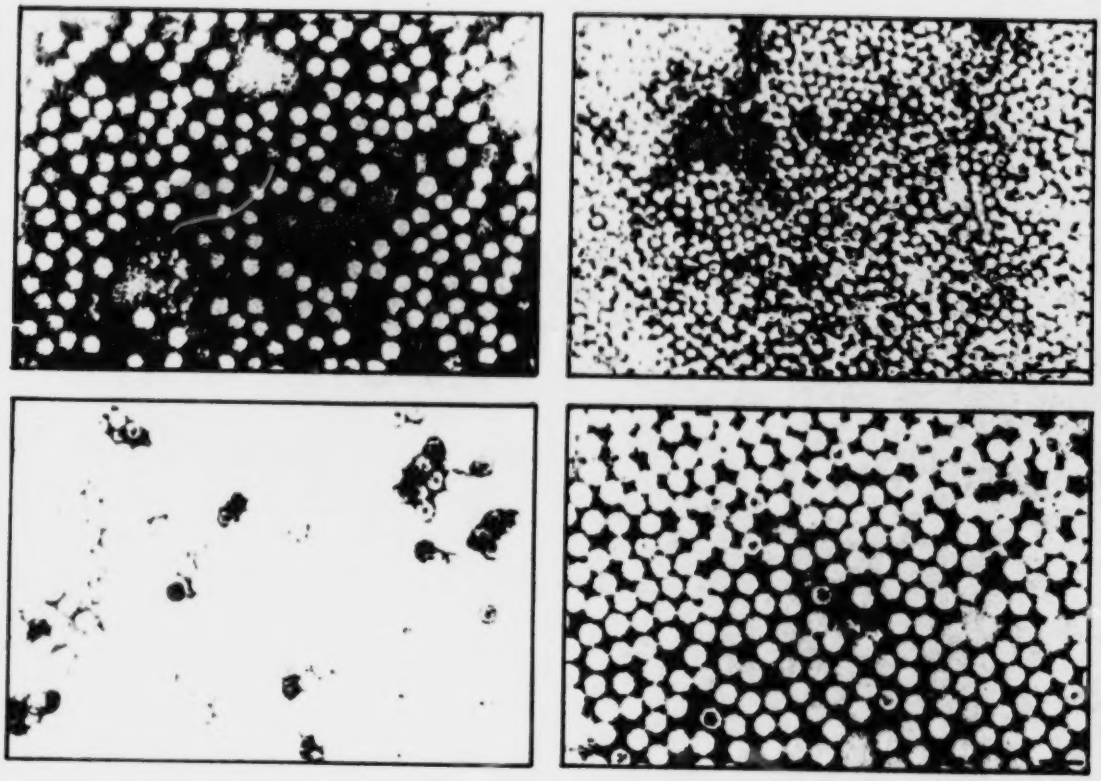
Dr T Senboku	-	Division of Plant Pathology & Microbiology Department of Agriculture Tropical Agricultural Research Center Ministry of Agriculture Forestry & Fisheries Yatabe Tsukuba Ibaraki 305 Japan
	-	and c/o Division of Plant Pathology & Microbiology Department of Agriculture Bangkhen, Bangkok 9 Thailand
Dr E Shikata	-	Laboratory of Plant Virology & Mycology Department of Botany Faculty of Agriculture, Hokkaido University Sapporo Japan
Dr R Suseno	-	Department of Plant Pests & Diseases Faculty of Agriculture Bogor Agricultural University Jalan Raya Pajajaran Bogor Indonesia
Dr T K Tan	-	Botany Department National University of Singapore Lower Kent Ridge Road Singapore 0511
Dr S Vora-Urai	-	Biology Department Chiang Mai University Chiang Mai 50000 Thailand
Dr C Y Yang	-	Thailand Outreach Programs/AVRDC 4th Floor, New Agricultural Building Kasetsart University Bangkhen, Bangkok 10900 Thailand
Dr Yeoh Hock Hin	-	Botany Department National University of Sngapore Lower Kent Ridge Road Singapore 0511

Observers

Mr C B Carpio	-	Philippine Coconut Authority Agricultural Research Branch Albay Research Center Banao, Guinobatan Albay 4908 Philippines
Mrs Asmah Haji Saman Dayang	-	Department of Agriculture Brunei
Miss P S Eng	-	Department of Botany National University of Singapore Lower Kent Ridge Road Singapore 0511
Mr S S Liau	-	Guthrie Research, Chemara Jalan Labu, Seremban Negeri Sembilan Malaysia
Dr Serudin bin Datu Setiawan Haji Tinggal	-	Department of Agriculture Brunei
Mr Y Sulyo	-	Segunung Sub Research Institute for Horticulture P O Box Pacet Cianjur West Java Indonesia
Mr T A Yong	-	Agriculture Section Sembawang Field Experimental Station Sembawang Road Singapore 2276
Miss Y K Fong	-	Singapore Botanic Gardens Cluny Road Singapore 1025



Sitting (L to R): J S Imperial, D Choopanya, Asmah Hj Saman, A N Rao, C Lim, N Abdul Samad, R Suseno, M Raychaudhuri
Standing (L to R): S Green, S S Liau, Y Sulyo, S Attathom, C Yang, Serudin Hj Tinggal, C I Gonzales, K Balaraman, H H Yeoh, S Vora-Urai, T Senboku, C B Carpio, E Shikata, T K Tan



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